Nitrogen cycling in heathland ecosystems and effects of climate change

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This thesis is a product of helpful and committed laboratory staff, field assistants, supervisors and fellow students.

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A: The CLIMAITE field site with temperate heath *Deschampsia flexuosa* and *Calluna vulgaris* vegetation. Left: ladder for vertical placement on the 7 m. diam. octagon (center) for safe-keeping the plots as non-disturbed. The right hand tall bar has the water exclusion curtain. The front low bar has the warming curtain.

Part I: manuscript nr 1 submitted to Plant and Soil:
'Uptake of pulse injected nitrogen by soil microbes and mycorrhizal and non-mycorrhizal plants in a species-diverse subarctic heath ecosystem'
L. C. Andresen, S. Jonasson L. Ström and A. Michelsen

Part II: manuscript nr 2 submitted to Soil Biology and Biochemistry:
'Free amino acid and ammonium uptake in temperate heathland vegetation and soil microorganisms under influence of enhanced soil tannic acid'
L. C. Andresen, A. Michelsen, S. Jonasson and L. Ström

Part III: manuscript nr 3:
'Plant nutrient mobilization in temperate heathland responds to drought, elevated temperature and CO_2'
L.C. Andresen, A. Michelsen, S. Jonasson, I.K. Schmidt, T. Mikkelsen, P. Ambus and C. Beier

Part IV: manuscript nr 4:
'Glycine acquisition in temperate heath vegetation and soil microorganisms is influenced by elevated temperature, CO_2 and drought'
L.C. Andresen, A. Michelsen, S. Jonasson, C. Beier and P. Ambus

Summary

Terrestrial ecosystems are currently exposed to climatic and air quality changes with increased atmospheric CO_2, increased temperature and periodical droughts. At a temperate heath site the combined effects of warming, increased atmospheric CO_2 and summer drought was investigated in a unique full factorial *in situ* experiment (CLIMAITE). The climate change treatments started October 2005 and consisted of increased temperature (T), extended summer drought (D), increased atmospheric CO_2 and all combinations of these treatments (TD, TCO_2, DCO_2 and $TDCO_2$).

In this thesis, responses in soil inorganic and microbial nutrient concentration were investigated after one year of climate change treatment. Additionally, top soil net mineralization, immobilization and leaf litter decomposition was investigated through the winter half year separately below *Calluna* and *Deschampsia* plants, and acquisition of organic nitrogen in plants and soil microorganisms was assessed.

After one year of treatments, warming increased microbial N, C and P and decomposition of leaf litter below *Calluna* plants. In *Deschampsia* soil the net nitrification rate decreased significantly in response to drought, by contrast, an increase was observed in *Calluna* soil. Drought reduced leaf litter decomposition for both species.
In warmed plots an early senescence was observed with effects on green *Deschampsia* biomass, on *Deschampsia* root nitrogen concentration and on acquisition of ^{15}N from glycine.

In this thesis, experiments using the stable isotopes ^{15}N and ^{13}C as tracers of ammonium and amino acid acquisition by plants and soil microorganisms suggest directions of the short term competition at two dwarf shrub heaths, one with sub-arctic climate and one with temperate climate during spring and fall. Soil microorganisms acquired the largest amount of the added nitrogen sources compared to plants at both heath types. At both heaths, plants preferred the inorganic ammonium, yet all nitrogen forms were acquired by both plants and soil microorganisms. At the temperate heath, soil microorganisms acquired the ^{15}N ^{13}C labeled amino acids (glycine, glutamic acid and phenylalanine) as intact compounds, and both dominant plant species showed indications of phenylalanine acquisition as intact

compounds. The thesis consists of an introduction collecting the most important findings from the four manuscripts.

Sammenfatning

Over vore terrestriske økosystemer er der til stadighed klimatiske forandringer med øget CO_2, forhøjet temperatur, og periodevis forlænget tørke. På tempereret hede I Danmark undersøges de kombinerede effekter af opvarmning, forhøjet CO_2 og forlænget sommer tørke i et unikt fuld faktorielt *in situ* forsøg (CLIMAITE). Klimabehandlingerne startede I oktober 2005 og består I forhøjet temperatur (T), forlænget sommertørke (D), forhøjet CO_2 og alle kombinationer af disse behandlinger (TD, TCO_2, DCO_2 og $TDCO_2$).

I dette ph.d arbejde undersøges forandringer i jords uorganiske og organiske næringsstofsammensætning og mikrobiel biomasse efter et års kontinuerlig klimaforandring. Desuden undersøges gennem vinterhalvåret nedbrydningsprocesser i det øverste jordlag som mineralisering, nitrifikation og immobilisering samt nedbrydning af dødt bladmateriale, adskilt for de to dominerende plantearter *Calluna vulgaris* og *Deschampsia flexuosa*. Optag af uorganisk og organisk næring undersøges i planter og jordbunds mikroorganismer.

Klimaforandringerne forøgede den mikrobielle biomasse (N, C og P) og blad nedbrydning under *Calluna* ved forhøjet temperatur, og *Deschampsia* udviste prematur senescens med mindsket grøn biomasse og øget rod N koncentration. Effekter af opvarmning blev dog ofte modvirket når tørke og CO_2 kombineredes med opvarmning. I jord under *Descampsia* faldt netto nitrifikations raten efter øget sommer tørke mens den steg I jord under *Calluna*. Tørke mindskede desuden blad nedbrydningen for begge arter.

I dette ph.d. arbejde undersøges optag af næringsstofferne ammonium og aminosyrer i plante og jordbunds mikroorganismer ved anvendelse af de stabile isotoper ^{15}N og ^{13}C som sporstoffer. Dette vægter korttids konkurrecen på to dværgbusk heder, en med subarktisk klima og en med tempereret klima tidligt og sent på året. Mikroorgaismer optog den største part af det tilførte nitrogen på begge heder. På begge heder foretrak planter ammonium, dog optages alle kvælstofformer af bade planter og mikroorganismer. På tempereret hede optog mikroorganismerne aminosyrere glycine, glutamin syre og phenylalanine som hele molekyler og begge dominerende platearter viste tegn på optag af intakt pheylalanin.

Afhandlingen består af en itroduktion der samler de fire udarbejdede manuskripter.

Nitrogen cycling in heathland ecosystems and effects of climate change

Knowledge of terrestrial ecosystem cycling of nitrogen is building from investigations and experiments through decades with curious and laborious exploration of soil and plant interactions (Sorensen *et al.*, 2008b; Sorensen *et al.*, 2006; Schmidt *et al.*, 2002; Emmett *et al.*, 2004; Jonasson *et al.*, 1993; Aerts & Chapin III, 2000; Paul & Clark, 1996). The openness of the heathland ecosystem with nitrogen deposition and nitrous gas emissions emphasizes the vulnerability of the mutualism (Sorensen *et al.*, 2006). Nitrogen limitation is often announced as controlling plant primary production at the heath (Aerts & Chapin III, 2000; Riis-Nielsen *et al.*, 2005). Consequently, competition for inorganic and organic nitrogen sources between plant species and between plants and soil microorganisms is key to the coexistence of these organisms in seasonal and dynamic patterns (Nordin *et al.*, 2004; Clemmesen *et al.*, 2008).

Disregarding nitrogen deposition and emissions, production of the inorganic nutrients: nitrate and ammonium and abundance of released amino acids in the soil solution sets the frame for biomass production. Amino acids in the soil function both as nitrogen sources and as labile carbohydrate substrates for soil microorganisms (Ström & Christensen, 2007; Vestergård *et al.*, 2008). The ability of the competing organisms to acquire these nutrients reflects the strategy and differentiated niches of the organisms.

Nutrient concentrations in the soil solution does not necessarily represent a concomitant high flux of the compound e.g. NO_3^-, NH_4^+ or amino acids, and a measured low concentration of e.g. amino acids may 'hide' a high flux of these compounds (Weintraub & Schimel, 2005b; Kielland *et al.*, 2007). Hence, nutrient flux parameters, such as enzyme concentration in the soil, nitrification and mineralization rates or use of nutrient labels with stable isotopes to trace short-term acquisition, dynamically describe importance of nutrient compounds in the ecosystem cycling.

In this thesis, experiments using the stable isotopes ^{15}N and ^{13}C as tracers of ammonium and amino acid acquisition by plants and soil microorganisms suggest directions of the short

term competition at two dwarf shrub heaths, one with sub-arctic climate and one with temperate climate during spring and fall. We expected:

- That both microorganisms and plants would be able to acquire N in both the added inorganic and organic forms.
- Soil microorganisms would acquire the largest amounts of the added nitrogen sources compared to plants at both heath types.

At the subarctic heath, plants overall preferred the inorganic ammonium while soil microorganisms preferred the organic amino acids glycine and glutamic acid, yet all nitrogen forms were acquired by both plants and soil microorganisms (manuscript 1). At the temperate heath, soil microorganisms showed no preferences of nitrogen form, hence ammonium and the amino acids: glycine, glutamic acid and phenylalanine were acquired equally (manuscript 2). Soil microorganisms acquired the ^{15}N ^{13}C labeled amino acids as intact compounds, and both plant species showed indications of phenylalanine acquisition as intact compounds. Both dominant plant species *Calluna vulgaris* and *Deschampsia flexuosa* showed preference of ammonium over the amino acids (manuscript 2).

Terrestrial ecosystems are currently exposed to climatic and air quality changes with increased atmospheric CO_2, increased temperature and periodical droughts. According to extrapolations and models developed by IPCC, the air temperature may increase by 0.1 ˚C for each following decade and the CO_2 concentration of the atmosphere will increase with an amount depending on stabilization scenario. Furthermore, precipitation will alter with expected extended summer drought periods in Denmark (IPCC, 2007); (Danish Meteorological Institute, 2008). At the temperate heath site the combined effects of warming, increased atmospheric CO_2 and summer drought on the soil processes was investigated in a unique full factorial *in situ* experimental set up (CLIMAITE). The climate change treatments started October 2005 and consisted of increased temperature (T), extended summer drought (D), increased atmospheric CO_2 and all combinations of these treatments (TD, TCO_2, DCO_2 and $TDCO_2$) (Mikkelsen *et al.*, 2008).

In this thesis, responses in soil inorganic and microbial nitrogen concentrations were investigated after one year of climate change treatments (manuscript 3). Additionally, top soil net mineralization and microbial N immobilization and leaf litter decomposition was

investigated through the winter half year separately below *Calluna* and *Deschampsia* plants. We expected that (manuscript 3):

- Biological processes would be stimulated by warming (T) leading to increased net rates of nitrification, mineralization and decomposition as well as increased microbial C, N and P.
- Decomposing microorganisms would be water limited by the drought treatment (D) leading to reduced nitrification, mineralization and decomposition in response to drought.
- Plant presence will induce microbial immobilization and acquire mineralized nitrogen.

Inclusion of live *Calluna* or *Deschampsia* plants in the soil incubations revealed differentiated responses in mineralization, microbial immobilization and plant mobilization of nitrogen. Warming increased microbial N, C and P at 0-5 cm depth and decomposition of leaf litter below *Calluna* plants. The effects of warming were often counteracted when combined with both CO_2 and drought. Net mineralization of N and P was significantly affected by the climate change treatments. In *Deschampsia* soil the net nitrification rate decreased significantly in response to drought, by contrast, an increase was observed in *Calluna* soil. Drought reduced leaf litter decomposition for both species. Plant presence increased the microbial immobilization, suggesting a plant root exudation priming of the rhizosphere. Warmed plots with lower DOC concentrations had lower mineralization rates, also suggesting a carbohydrate limitation of the microbes (manuscript 3).

Root uptake kinetics are enhanced by warming, and the acquisition may increase by changed root transport properties for NH_4^+ (Clarkson & Warner, 1979; Pike & Berry, 1980). Furthermore, NO_3^- uptake capacity is highly modulated by the N status of the roots or the whole plant (Bassirirad, 2000). Root biomasses, depth distribution and root morphology respond differentially to warming (Björk *et al.*, 2007). Consequently, the acquired N pool of the plant roots in response to warming is a combined effect of root biomass, nutrient status and root growth responses combined with the acquisition physiology parameters. Responses in root nutrient uptake to elevated CO_2 is highly variable, reflecting e.g. differential responses in plant growth and nutrient status, while plant processes such as water-use efficiency, photosynthetic rate (Ehleringer, 2005), tissue N-concentration and labile

carbohydrates show consistent responses to elevated CO_2 (Bassirirad, 2000). Responses in root nutrient uptake to elevated CO_2 is highly variable, reflecting e.g. differential responses in plant growth and nutrient status, while plant processes such as water-use efficiency, photosynthetic rate, tissue N-concentration and labile carbohydrates show consistent responses to elevated CO_2 (Bassirirad, 2000). Carbohydrate exudation by plant roots may respond to climate change in the same direction as photosynthesis and plant production (Rinnan *et al.*, 2005; Albert *et al.*, 2005; Ehleringer, 2005). Hence, elevated temperature and CO_2 may increase soil concentrations of e.g. glycine. In this experiment we investigated the acquisition and partitioning of glycine between plants and soil microorganisms.

In an *in situ* labeling experiment with ^{15}N ^{13}C glycine in the climate treated plots we expected (manuscript 4):

- warming to promote biological activity, by increasing root ^{15}N uptake
- elevated CO_2 to increase plant biomass

Furthermore, changes in abundance of plant nutrients (nitrate or ammonium) in the soil solution would affect root biomass or N concentration:

- an increase in nitrate concentration would cause a smaller root biomass and vice versa

Nitrogen pools cycling at the subarctic heath

An investigation of ecosystem nitrogen pools and plant and microbial inorganic and organic nitrogen acquisition was investigated in a short term experiment (manuscript 1). Furthermore, long-term (11 years) ecosystem retention of nitrogen was assessed.
At a mesic low productive subarctic heath (Michelsen *et al.*, 1998; Michelsen *et al.*, 1999) the vegetation was species diverse and dominated by deciduous (126 g m^{-2} aboveground) and evergreen (170 g m^{-2}) dwarf shrubs with a low cover of graminoids (19 g m^{-2}), other herbs (14 g m^{-2}) and cryptogams (21 g m^{-2}) (manuscript 1). The plant species had ericoid-, ecto- and arbutoid mycorrhiza or were non-mycorrhizal (Michelsen *et al.*, 1998; Clemmesen *et al.*, 2006; Olsrud *et al.*, 2004).

The distribution of nitrogen between the ecosystem pools at the subarctic heath field site from top canopy down to 10 cm depth was (manuscript 1):

	gN m^{-2}
NH_4^+-N	0.56 ± 0.05
Amino acid N ×10^{-6}	296 ± 5
DON	2.73 ± 0.23
DTN	3.29 ± 0.27
MicN	10.93 ± 0.90
Plant N	29.0 ± 0.6

Table A: Nitrogen pools at the sub arctic heath field site (manuscript 1) NH_4^+-N, amino acid nitrogen, dissolved organic nitrogen (DON), dissolved total nitrogen (DTN), microbial nitrogen (MicN), plant nitrogen.

These pool sizes were in line with another investigation at a near by dry heath site, also with NO_3^- concentrations below detection limit (Sorensen *et al.*, 2008a; Schmidt *et al.*, 2002; Michelsen *et al.*, 1999).

Acquisition of nitrogen was investigated with fully stable isotope ^{15}N labeled compounds injected in situ at the subarctic heath site. 21 days after addition of (each 0.130 gN m^{-2}) ^{15}N ammonium, glycine or glutamic acid in 1 cm depth, the recovery of the ^{15}N label at the subarctic heath was (manuscript 1):

	% ^{15}N recovery ^{15}N ammonium	**% ^{15}N recovery ^{15}N glycine**	**% ^{15}N recovery ^{15}N glutamic acid**
DTN	4.2 ± 1.3	3.4 ± 0.3	4.4 ± 0.7
MicN	23.7 ± 3.3 (B)	38.6 ± 3.5 (AB)	46.6 ± 12.7 (A)
Total soil	46.3 ± 13.8	57.4 ± 10.3	69.8 ± 16.3
Plant (green/leaf)	2.0 ± 0.4 A	1.2 ± 0.2 AB	0.5 ± 0.1 B

Table B: ^{15}N recovery of added label in plants and dissolved organic N, microbial N and total soil 21 days after labeling at the subarctic heath field site (manuscript 1).

Hence, the microbial acquisition of each of the added labels was larger than the plant acquisition. This was in line with what has been found in other investigations using the same methodology (Schimel & Chapin, 1996; Hofmockel *et al.*, 2007; Sorensen *et al.*, 2008a; Sorensen *et al.*, 2008b; McKane *et al.*, 2002). Furthermore microorganisms by tendency preferred glutamic acid, while plants significantly preferred ammonium, se manuscript 1. This suggested microbial preference for organic nitrogen may be site specific, however plant preference of inorganic nitrogen seems to be more general across ecosystems (Nordin *et al.*, 2004; Sorensen *et al.*, 2008a; Clemmesen *et al.*, 2008; Kielland *et al.*, 2006; Harrison *et al.*, 2008) and manuscript 2).

In a sampling of the ^{15}N labeled plots 11 years after the original ^{15}N labeling, the same pools were investigated following the same methodology of the first study in manuscript 1. No significant effects of the original labeled N form or of the original depth of labeling was found, as was the case after one year in a study using NO_3, NH_4 and glycine at a more dry heath (Sorensen *et al.*, 2008b). After 11 years of natural ecosystem cycling of the originally added ^{15}N label the average ^{15}N recovery of the label added in 1 cm depth at the subarctic heath was:

	cm depth	**% ^{15}N recovery**
Plant abovegr		1.4 ± 0.1
Plant litter		1.8 ± 0.2
Coarse roots	0-5	1.7 ± 0.3
	5-10	0.1 ± 0.1
	10-15	0.0
Fine roots	0-5	2.4 ± 0.4
	5-10	0.1 ± 0.0
	10-15	0.0
Dissolved Total N	0-5	0.1 ± 0.0
	5-10	0.0
	10-15	0.0
Microbial N	0-5	5.0 ± 0.9
	5-10	0.8 ± 0.2
	10-15	0.1 ± 0.1
Total soil N	0-5	36.8 ± 3.6
	5-10	4.2 ± 0.8
	10-15	1.0 ± 0.2
Total ecosystem		49.5 ± 5.7

Table C: ^{15}N recovery of added label in plants and dissolved organic N, microbial N and total soil 11 years after labeling at the subarctic heath field site.

This is the first study to investigate long term retention and cycling of added stable isotope ^{15}N nitrogen. The total ecosystem (total soil plus plant fractions) ^{15}N recovery, reflects a leaching of the added ^{15}N of about 50 % through the period. Hence, this rather large long-term retention of added nitrogen is informative when assessing the ecosystem vulnerability to anthropogenic nitrogen deposition.

The temperate heath: nitrogen pools and cycling

The field site of the investigation was at Brandbjerg (55º53'N 11º58'E) just next to the climate treated plots a hilly nutrient poor sandy deposit with a dry heath/grassland ecosystem dominated by *Deschampsia flexuosa* (460 g m^{-2} DW (above plus below ground)) and *Calluna vulgaris* (715 g m^{-2} DW (above plus below ground)) and with a low cover of other herbs and grass species, and an open moss cover beneath the canopy of vascular plants. The average precipitation per year was about 600 mm and the average temperature was 8° C. The N deposition is around 1.25 gN m^{-2} $year^{-1}$ (www.dmi.dk, 2005; Mikkelsen *et al.*, 2008), and manuscript 2). The distribution of nitrogen between the ecosystem pools at the temperate heath from top canopy down to 5 cm depth was (manuscript 2):

	gN m^{-2}
NO_3^--N	0.001
NH_4^+-N	0.008
Amino acid N $\times 10^{-6}$	0.001
DON	0.065
MicN	0.831
Plant N	13.4

Table D: Nitrogen pools at the temperate heath field site, May 2005 (manuscript 2) NO_3^--N, NH_4^+-N, amino acid N, dissolved organic N (DON), microbial N (MicN), plant N (above and belowground, all species).

Other studies of similar heath ecosystems using the same methodology have shown similar pool sizes (Sowerby *et al.*, 2005; Jensen *et al.*, 2003; Schmidt *et al.*, 2004).

At the temperate heath field site, the dynamics of the nitrogen cycling was investigated in soil incubations below the two dominant plant species in buried bags (Eno, 1960; Jonasson *et al.*, 2006; Schmidt *et al.*, 2002) , yielding net nitrification, net mineralization, net dissolved organic N production and net microbial immobilization (manuscript 3) incubated through the winter half year (187 days) (manuscript 3):

Ambient climate treatment	***Calluna* soil**	***Deschampsia* soil**
Nitrification (ΔNO_3^--N) µg N g^{-1} SOM day^{-1}	-0.049 ± 0.083	0.224 ± 0.090
Mineralization (ΔNH_4^+-N) µg N g^{-1} SOM day^{-1}	0.786 ± 0.570	0.454 ± 0.296
DON production (ΔDON) µg N g^{-1} SOM day^{-1}	-0.679 ± 0.555	-0.374 ± 0.385
Immobilization (ΔMicN) µg N g^{-1} SOM day^{-1}	-0.066 ± 2.888	-0.750 ± 1.621

Table E: Net nitrification, net mineralization, net DON production and net microbial immobilization at the temperate heath field site, over winter 2006-2007 (187 days) (manuscript 3).

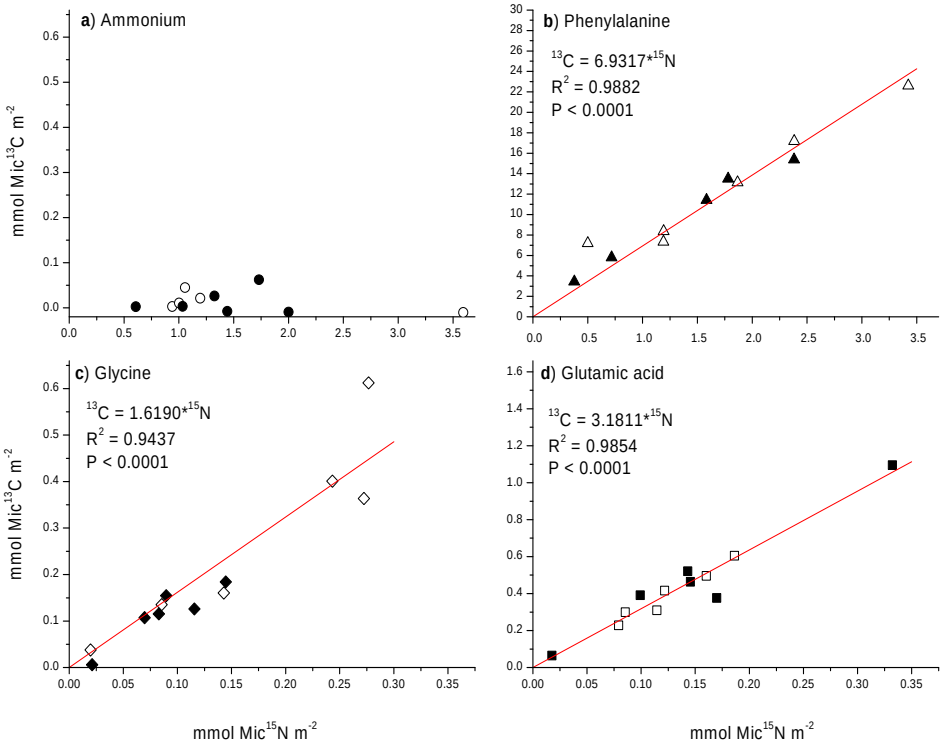
These ranges were comparable to nitrification and mineralization rates for other studies at *Calluna - Deschampsia* dominated heaths using the same methodology (Emmett *et al.*, 2004; Beier *et al.*, 2004).

The acquisition of ammonium and the amino acids glycine, glutamic acid and phenylalanine by plants and soil microorganisms were investigated *in situ* at the temperate heath field site with fully ^{15}N and ^{13}C labeled compounds. One day after labeling with the different nitrogen forms at the temperate heath during spring, the recovery of the ^{15}N labels were (manuscript 2):

	% ^{15}N recovery ^{15}N ammonium	**% ^{15}N recovery ^{15}N $^{13}C_2$ glycine**	**% ^{15}N recovery ^{15}N $^{13}C_5$ glutamic acid**	**% ^{15}N recovery ^{15}N $^{13}C_9$ phenylalanine**
DTN	0.6 ± 0.2	0.8 ± 0.1	1.3 ± 0.4	1.0 ± 0.6
Microbial N	46.7 ± 15.3	52.0 ± 13.2	37.4 ± 5.1	52.8 ± 12.7
Total soil	87.1 ± 17.1	76.6 ± 24.2	88.6 ± 20.3	86.3 ± 8.7
Calluna	3.9 ± 1.1	0.7 ± 0.2	0.6 ± 0.2	0.9 ± 0.3
Deschampsia	3.9 ± 1.1	1.2 ± 0.4	1.3 ± 0.4	0.8 ± 0.3

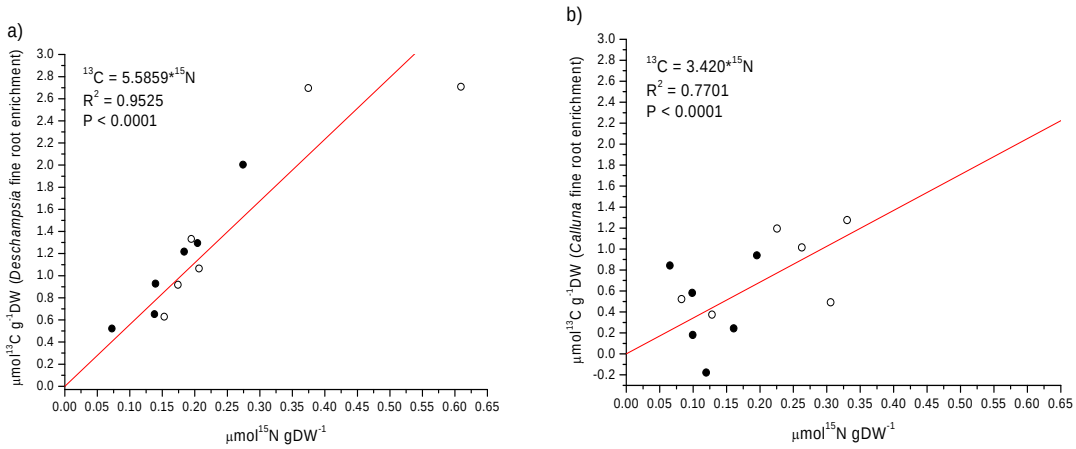
Table E: acquisition of ^{15}N from the labels ammonium, glycine, glutamic acid and phenylalanine by whole plants of *Deschampsia flexuosa* and *Calluna vulgaris* and dissolved total nitrogen (DTN), microbial nitrogen and in total soil, one day after labeling at the temperate heath (manuscript 2).

The soil microbial acquisition of the amino acids was as intact compounds, as seen from the ^{13}C to ^{15}N ratios (manuscript 2):



B: Enrichment of ^{13}C and ^{15}N in soil microorganisms from the labels: a) ^{15}N-ammonium b) ^{15}N $^{13}C_9$-phenylalanine c) ^{15}N $^{13}C_2$-glycine d) $^{15}N^{13}C_5$-glutamic acid one day after labeling at the temperate heath (manuscript 2).

Plant root acquisition of phenylalanine was also found to be partly of non-mineralized compounds, with the enrichment ^{13}C to ^{15}N ratios in *Deschampsia* roots of 5.6 and in *Calluna* roots of 3.4 compared to 9 as the ^{13}C to ^{15}N ratio of the added phenylalanine (manuscript 2).



C: Enrichment of ^{13}C and ^{15}N in a) *Deschampsia flexuosa* fine roots and b) *Calluna vulgaris* fine roots from ^{15}N $^{13}C_9$-phenylalanine one day after labeling at the temperate heath (manuscript 2).

These results (manuscript 2) of intact acquisition of the large amino acid in an *in situ* experiment are additional evidence of possible plant short circuiting of the soil mineralization cycle (Schimel & Bennett, 2004; Kielland *et al.*, 2007; Kielland *et al.*, 2006; Nordin *et al.*, 2004; Sorensen *et al.*, 2008a; Mikkelsen *et al.*, 2008; Andresen & Michelsen, 2005). Furthermore, the large acquired amount of the amino acids contributes to the discussion of organic nitrogen as potentially important nutrient pools of ecosystems, in spite of the rather low water extractable free amino acid pool at the field site.

During fall 2006, acquisition of $^{15}N^{13}C$-glycine by plants and soil microorganisms at the temperate heath field site was investigated in the field plots of the climate manipulation experiment, with elevated temperature, elevated CO_2 and summer drought, to evaluate effects of climate change on organic nitrogen acquisition by the competing heathland organisms.

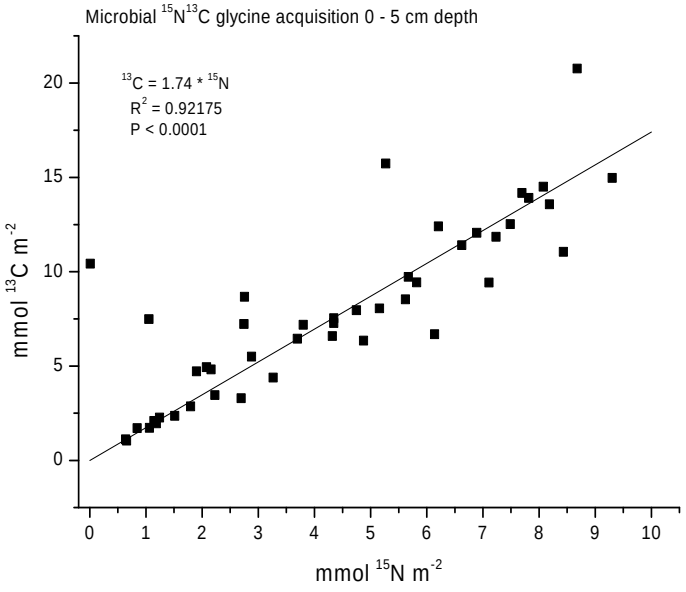
Following the same methodology as in manuscript 2, one day after glycine addition at the temperate heath during fall the ^{15}N recovery of the added label was (manuscript 4):

	cm depth	A	D	T	TD	CO2	DCO2	TCO2	TDCO2
Deschampsia		1.4 ± 0.4	2.5 ± 1.1	2.5 ± 1.0	2.0 ± 0.6	3.6 ± 0.7	3.4 ± 1.0	2.0 ± 0.4	2.5 ± 0.6
Calluna		0.8 ± 0.4	1.3 ± 0.5	1.4 ± 0.7	0,7 ± 0.2	0.8 ± 0.2	1.3 ± 0.4	0.6 ± 0.1	0.7 ± 0.3
Microbial N	**0-5**	35.7±13.7	54.5±15.3	89.1±49.1	36.9±10.7	62.3±16.0	59.3±4.0	56.5±13.6	110.2±63.6
	5-10	10.7±5.6	10.7±4.6	8.1±3.2	6.3±2.2	10.9±4.8	5.7±2.8	2.9±1.1	8.9±3.2
	10-15	3.4±2.2	1.9±1.7	0.1±0.1	0.7±0.4	1.1±1.1	0.6±0.4	0.4±0.3	0.8±0.6
DTN	**0-5**	0.13±0.08	0.03±0.02	0.03±0.01	0.38±0.37	0.10±0.05	0.10±0.09	0.05±0.03	0.09±0.05
	5-10	0.00±0.00	0.01±0.01	0.16±0.13	0.03±0.03	0.02±0.01	0.00±0.00	0.01±0.00	0.03±0.02
	10-15	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.06±0.04	0.00±0.00	0.00±0.00	0.00±0.00

Table F: acquisition of ^{15}N from glycine by whole plants of *Deschampsia flexuosa* and *Calluna vulgaris* and dissolved total nitrogen (DTN), microbial nitrogen, one day after labeling at the temperate heath in the climate treated plots (fall). A: ambient, D: drought, T: temperature, CO2: elevated CO_2 (manuscript 4).

Hence, both during spring and autumn the soil microorganisms acquire a much larger amount of the added nitrogen than do the plants. Also at this late season labeling, plants preferred the inorganic nitrogen source (Andresen & Michelsen, 2005).

Additionally, soil microorganisms acquired the added glycine as intact compounds at the autumn labeling, with a ^{13}C to ^{15}N ratio of 1.7 (manuscript 4):



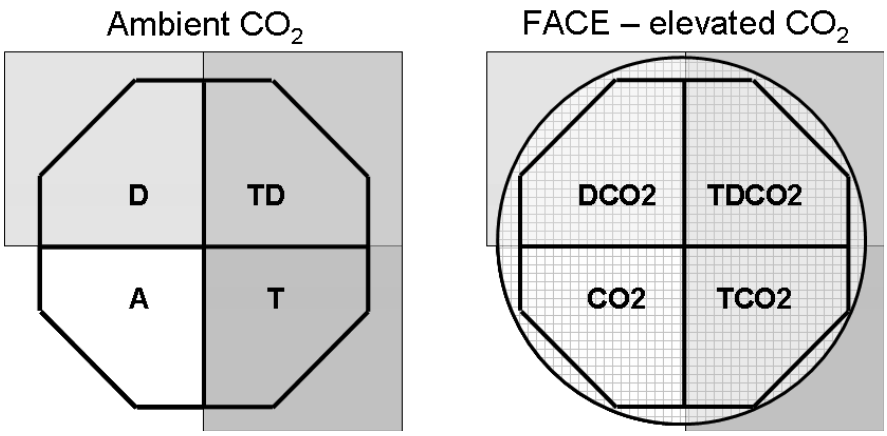
D: Enrichment of ^{13}C and ^{15}N in soil microorganisms from ^{15}N $^{13}C_2$-glycine, one day after labeling at the temperate heath (fall) all climate treatments (manuscript 4).

In conclusion from manuscript 1,2 and 4: at both heath types and at the temperate heath at two times during the season, soil microorganisms win the short term competition over an

added nitrogen pulse; plants prefer to acquire inorganic nitrogen and soil microorganisms acquire the amino acids as intact compounds.

Climate change effects on nitrogen cycling

At the temperate heath site, the combined effects of warming, increased atmospheric CO_2 and summer drought on the soil processes was investigated in a full factorial in situ experimental set up. The climate manipulations started October 2005, and consisted of increased temperature (T), extended summer drought (D), increased atmospheric CO_2 and all combinations of these treatments (TD, TCO_2, DCO_2 and $TDCO_2$), all with a replication of 6. The study plots consisted of 12 octagons each 7 m in diameter, comprising 4 plots in a split plot design with the treatments drought or elevated temperature solely or in combination, and a non-warmed, non-drought plot.



E: Schematic design of climate treatments (CLIMAITE) adapted from Mikkelsen et al. 2008

The temperature was increased by passive nighttime warming by means of low automatic curtains that were automatically removed during rain events. The precipitation was altered also with automatic curtains that automatically unfolded during rain events. The atmospheric CO_2 was increased with pipe fumigation as in a regular FACE experiment, and with a feed back control system linked to wind speed and wind direction. The temperature increase of the soil in 2 cm depth was around 1˚C, the increased CO_2 concentration in the air was 510 ppm. The drought period started in late June 2006 and continued for 5 weeks until early August when soil water reached c. 5 vol% water in the top 20 cm of the soil. For further

information about the experimental design of the multifactor set up, see Mikkelsen et al 2008.

F: Area photo of the CLIMAITE field site at Brandbjerg the 12 circles represent the 12 Octagons with each 4 plots. © Google ™ 2007.

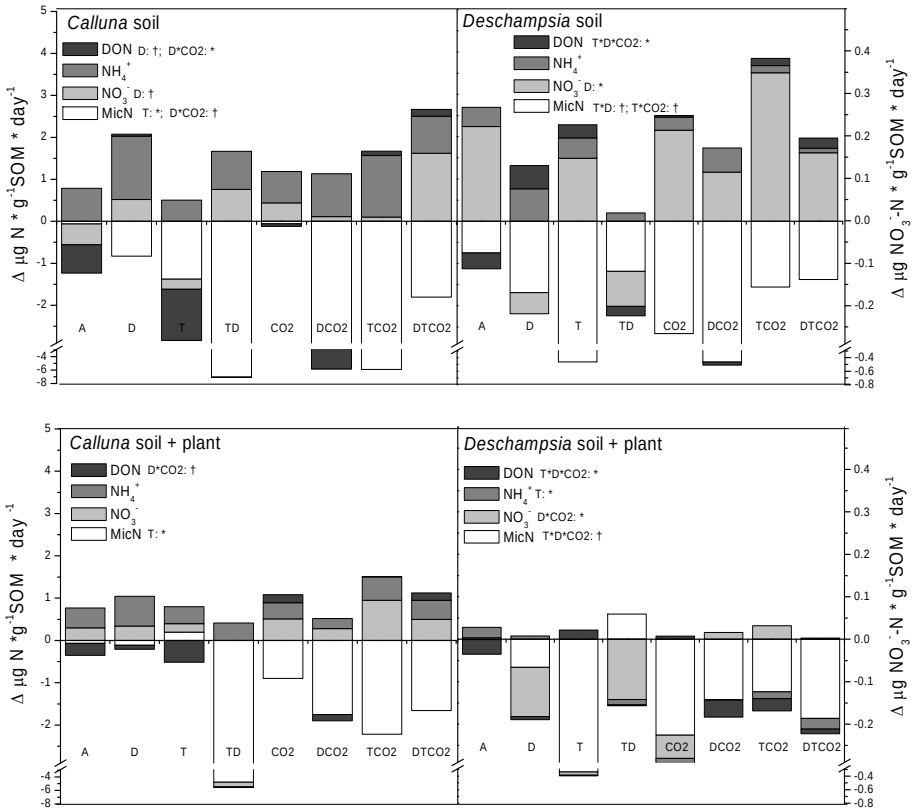
Soil N and P mineralization, microbial immobilization and decomposition were investigated in order to reveal climate change effects on nutrient cycling. This study was made for the two dominant species separately, hence leaf litter from the two species and soil from the two species was incubated separately in litter bags and buried bags placed in the climate

treatments. The buried bags were additionally also incubated in a version with presence of plants (manuscript 3).

The two soil types below *Calluna* and below *Deschampsia* had different patterns of nutrient cycling, as expected from other studies investigating mineralization in soil below different plant species (van Vuuren *et al.*, 1992; van der Krift & Berendse, 2001; Gill *et al.*, 2006). In other investigations of temperate heathlands, N mineralization in soil below grasses and decomposition of grass litter was faster than for *Calluna* (van Vuuren *et al.*, 1992; van Vuuren *et al.*, 1993). Hence, a faster N cycling and a potentially stronger response to climate changes in soil below *Deschampsia* compared to soil below *Calluna*, may potentially control changes of the vegetation cover (van Vuuren *et al.*, 1992; Emmett *et al.*, 2004; Schmidt *et al.*, 2004; Weintraub & Schimel, 2005a).

In *Deschampsia* soil, net nitrification and litter decomposition decrease in response to drought, hence, drought works as suppressor of nitrogen cycling in the *Deschampsia* soil. *Calluna* soil responded to D with decreased nitrification and leaf litter decomposition, suggesting an opposite response of the *Calluna* soil-plant system to D (manuscript 3).

Pre-incubation differences were observed in the initial microbial biomass C, N and P pool increases in response to T, in consistence with other warming manipulations (Sowerby *et al.*, 2005; Schmidt *et al.*, 2002). In addition to this, the microbial N immobilization and SOM decomposition decreased and the leaf decomposition increased in response to T. In other investigations at temperate heaths, the natural gradient of soil temperature was the best predictor of soil respiration and litter decomposition (Emmett *et al.*, 2004). The initially smaller amount of DOC (total dissolved organic carbon) in warmed plots occurred together with larger microbial biomass, but still, mineralization in the successive incubations decreased. Hence, we suggest that the soil mineralization processes require an ongoing carbohydrate supply for instance by plant root exudation. The decreased DOC concentration it-self and the slower SOM decomposition and mineralization in our warmed plots may be a consequence of a shift from labile to recalcitrant carbon sources (Biasi *et al.*, 2005; Bengtson & Bengtsson, 2007).



G: Changes in soil nitrogen pools: nitrification rate (ΔNO_3^--N, right 2nd axis), mineralization rate (ΔNH_4^+-N, left 2nd axis) and dissolved organic N production rate (ΔDON, left 2nd axis) and microbial N immobilization rate (ΔMicN, left 2nd axis) in units per g soil organic matter (SOM) per day, after incubation for a half year. Four variations of incubations: *Calluna* soil and *Deschampsia* soil, with no plant or with plant. Statistical significant effects from proc mixed model analysis of variances for the main effects: D, T and CO2 and the interactions D*T, D*CO2, T*CO2 and D*T*CO2 is indicated as follows: * indicates P < 0.001; ** indicates P < 0.01; *: P < 0.05; †: P < 0.1 (manuscript 3).**

From the glycine labelling experiment increased nitrogen acquisition by *Deschampsia* in warmed and in CO_2 treatments was suggested (manuscript 4). Hence, when investigated in the autumn, warming resulted in increased *Deschampsia* root nitrogen acquisition and increased microbial biomass in *Calluna* soil. The possibly earlier senescence, seen by a smaller green *Deschampsia* leaf biomass may also cause the larger N concentration and ^{15}N acquisition, also being a phenomena of late season nitrogen acquisition and storage (Andresen & Michelsen, 2005).

The climate change factors significantly caused physiological-ecological changes in the temperate heathland ecosystem. Soil microorganisms acquired the largest part of the added glycine and acquired intact compounds with no significant effects of treatment. *Deschampsia* and *Calluna* plants also acquired glycine, with no proof of intact acquisition. *Deschampsia* fine root biomass decreased in warmed plots reflected by larger nitrate concentration in the sub-soil. Large *Deschampsia* plant root ^{15}N acquisition in T and in CO_2 plots met our hypothesis of promoted plant N demand, when plant biomass increased, but this was a non-additive effect. *Deschampsia* green leaf biomass decreased in warmed plots but not when CO_2 was added, and *Calluna* green to coarse branch increased in warmed plots and in elevated CO_2 plots, but not when these treatments were combined. Hence, the responses to simulated increased root exudation in form of ^{15}N $^{13}C_2$-glycine were significant and non-additive (manuscript 4). This states that to fully investigate climate change effects on ecosystem nitrogen cycling, it is important for the reliability of the conclusions to control temperature, atmospheric CO_2 and precipitation patterns in multifactor *in situ* experiments.

This thesis completes investigations at two heathlands with subarctic and temperate climate. At both heath types amino acid abundance was investigated and acquisition of inorganic nitrogen in form of ammonium and organic nitrogen in form of different amino acids was investigated in plants and soil microorganisms. At both heath types all forms of nitrogen was acquired by plants and microorganisms with the largest acquisition by microbes. Soil microorganisms at the temperate heath acquired the amino acids as intact compounds. At the temperate heath *in situ* climate change treatments of elevated temperature, CO_2 and drought and all combinations in a full factorial design, revealed significant species specific and non-additive responses of the plant and soil processes. Soil net mineralization decreased below *Deschampsia* plants and tended to increase below *Calluna* plants in response to drought. Microbial biomass N and C increased in soil below *Calluna* plants in response to warming. Plant root nitrogen acquisition from ^{15}N $^{13}C_2$ labeled glycine increased as effect of increased plant biomass in response to warming and elevated CO_2, but this was non-additive. *Calluna* leaf tissue nitrogen concentration was diluted by elevated CO_2. These short term responses with different directions for the two dominant plant species are first from our multifactorial climate change *in situ* experiment ‘CLIMAITE’.

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Uptake of pulse injected nitrogen by soil microbes and mycorrhizal and non-mycorrhizal plants in a species-diverse subarctic heath ecosystem

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Abstract

^{15}N labeled ammonium, glycine or glutamic acid was injected into subarctic heath soil *in situ*, with the purpose of investigating how the nitrogen added in these pulses was subsequently utilized and cycled in the ecosystem. We analyzed the uptake of ^{15}N in mycorrhizal and non-mycorrhizal plants and in soil microorganisms in order to reveal probable differences in acquisition patterns between the two functional plant types and between plants and soil microorganisms. Following the label addition, the ^{15}N-enrichment in the soil water extracts of dissolved and microbial fractions and in total soil was analyzed after 21 days, and the ^{15}N-enrichment in leaves of plants species was analyzed after three, five and 21 days.

The soil microorganisms had very high ^{15}N recovery from all the N sources compared to plants. Microorganisms incorporated most ^{15}N from the glutamic acid source, intermediate amounts of ^{15}N from the glycine source and least ^{15}N from the NH_4^+ source. In contrast to microorganisms, all ten investigated plant species generally had higher ^{15}N uptake from the NH_4^+ source than from the amino acid sources. Non-mycorrhizal plant species had higher ^{15}N uptake than mycorrhizal plant species three days after labeling, while 21 days after labeling their uptake of amino acids was lower than and the uptake of $^{15}NH_4$ was similar to the mycorrhizal species. We conclude that the soil microorganisms were more efficient than plants in acquiring pulses of nutrients which, under natural conditions, occur after e.g. freeze-thaw and dry-rewet events. It also appears, that the mycorrhizal plants initially are less efficient than non-mycorrhizal plants in nitrogen acquisition, but in a longer term show larger nitrogen uptake than non-mycorrhizal plants.

Keywords: ammonium, amino acid, freeze-thaw cycle, mycorrhiza, ^{15}N, organic nitrogen, plant nitrogen uptake, root biomass.

Introduction

Dissolved organic carbon and nitrogen (DOC and DON) and inorganic N are released in pulses after e.g. freeze-thaw cycles in the soil (Larsen et al. 2002; Sharma et al. 2006), due to freezing-induced mechanical disruption of soil aggregates and lysis of plant root cells, fungal hyphae or bacteria. Like-wise, dry-rewet cycles in the top soil and other local disturbances such as rodent activity and trampling, may influence soil biota and organic matter turnover (Paul and Clark 1996). These pulses probably are important for the supply of nitrogen to the organisms, because in most arctic and subarctic terrestrial ecosystems nitrogen (N) is limiting for plant production, while carbon (C) is limiting the soil microbial biomass, (Illeris and Jonasson 1999; Michelsen et al. 1999; Schimel and Bennett 2004). The low amount of available nutrients contrasts the large pool of unavailable nutrients built into soil organic matter, resulting from temperature-limited litter and organic matter decomposition (Robinson et al. 1997; Rustad et al. 2001). The organic soil holds a microbial biomass with a large N pool approaching the pool size in the plants (Sorensen et al. 2008).

The DOC and DON pulses contain amino acids which are found in high concentrations in the soil (Kielland 1995; Michelsen et al. 1999; Schmidt et al. 1999; Sorensen et al. 2008), and are available for microbial uptake. Evidence is now accumulating, that also plants are able to acquire amino acids as intact molecules, using membrane amino acid transporters (Schimel and Chapin 1996; Näsholm et al. 1998; Williams and Miller 2001; Chalot et al. 2002; McKane et al. 2002; Bardgett et al. 2003; Nordin et al. 2004; Svennerstam et al. 2007). This implies that plants and microbes in these nutrient deficient soils may compete not only for mineralized inorganic N, but also for organic N, and plants, hence, may short-circuit the mineralization cycle (Schimel and Bennett 2004).

The relative importance of inorganic and organic N as sources for plants and microorganisms has become an issue in studies of competition between these organisms, which differ in life histories, surface to volume ratios of nutrient-absorbing tissue, and uptake and exudation mechanisms. The revealed niche differentiation of plant species in temporal (Jaeger et al. 1999; McKane et al. 2002; Grogan and Jonasson 2003; Andresen and Michelsen 2005) and spatial (McKane et al. 2002; Sorensen et al. 2008) N uptake patterns is complementary to the differentiated N form preference of species or organism groups (Kielland 1994; Lipson et al. 1999; Falkengren-Grerup et al. 2000; Cheng and Bledsoe 2004; Xu et al. 2006). Field studies in natural ecosystems with concomitant measurements of N uptake by soil microorganisms and plant species and their relative uptake of N from different sources are few (Schimel and Chapin 1996; Grogan and Jonasson 2003; Nordin et al. 2004; Hofmockel et al. 2007; Sorensen et al. 2008). Additionally, none of these have taken place in ecosystems with high plant species diversity and high potential for resource partitioning between species with different mycorrhizal associations. This makes generalizations regarding N acquisition by functional groups across different ecosystems difficult.

In this *in situ* experiment at a subarctic, mesic heath, we examined the plant and microbial acquisition patterns of nitrogen. ^{15}N-labelled NH_4^+, glycine or glutamic acid was injected into the soil as a pulse at one or two depths. Nitrogen derived from these sources was available to potentially competing plant species and microorganisms, both in the added form and also after possible transformation of the added N source by microbial immobilization, mineralization or adsorption. To reveal probable differences in acquisition patterns by plants and microorganisms, the

uptake of N from the added ^{15}N-labelled sources was analyzed by isotope ratio mass spectrometry of plants and microorganisms in soil extracts after chloroform fumigation.

We hypothesized that:

- nitrogen released in a pulse, which is likely to occur after e.g. freeze-thaw or dry-rewet events, would rapidly be acquired by plants and microorganisms;
- soil microorganisms and plants would differ in ^{15}N uptake from $^{15}NH_4^+$, ^{15}N glycine and ^{15}N glutamic acid sources, with the largest uptake by microorganisms;
- soil microorganisms would have larger uptake of ^{15}N from the amino acid sources than from NH_4^+;
- the ^{15}N uptake potential would differ among plant species, with higher acquisition from the amino acid sources by species with mycorrhizal associations than by non-mycorrhizal species;
- through time, plants would access an increasing amount of the added and mineralized ^{15}N label after turn-over in microorganisms;
- plant ^{15}N acquisition from the label injected at different depths would reflect depth distribution of fine roots.

Materials and methods

The site for the experiment was a low alpine/subarctic species-rich, mesic heath at the tree limit, about 450 m above sea level, near Abisko Scientific Research Station in northern Sweden. The soil has a pH of 7.1 and an organic profile depth of 15 - 20 cm (Jonasson et al. 1996; Michelsen et al. 1999). The soil organic matter (SOM) content was 83% of the soil DW.

Each N form was added as 0.1295 g N m^{-2} dissolved in water and injected into the soil with syringes on June 26, 1995. With a plot size of 20×20 cm and with 36 injection points, fixed as evenly distributed holes in a plate, each plot received 360 ml solution. The design was 8 plots with ^{15}N-ammonium chloride ($^{15}NH_4Cl$, 99 atom %) injected just below the soil surface at 1 cm depth, 8 plots with ^{15}N-ammonium chloride injected at 5 cm depth, 8 plots with ^{15}N-glycine ($^{15}NH_3CH_2COO$, 99 atom %) injected at 1 cm depth, 8 plots with ^{15}N-glycine injected at 5 cm depth and 4 plots with ^{15}N-glutamic acid ($^{15}NH_3CHCOOCH_2CH_2COO$, 98 atom % ^{15}N-L-glutamic acid) injected at 1 cm depth. The imbalance of the design was due to insufficient amount of ^{15}N glutamic acid available for injection at 5 cm depth.

Soil in the labeled plots (0 - 10 cm depth) was sampled on July 17 (after 21 days). Soil was additionally sampled on June 24 from five plots adjacent to the labeled plots for estimation of the natural concentrations of amino acids in the soil solution.

Following the injections, current year leaves (segments for *Equisetum*) of dominant and subdominant plant species were sampled on June 29 (after three days) and on July 17 (after 21 days). For the subsequent analysis, we used only current year leaves since they most clearly demonstrate recent N uptake and translocation to the nitrogen demanding photosynthesizing tissue. The plant species sampled for analyses were the graminoids *Carex vaginata* and *Carex parallela* (non-mycorrhizal), the forb *Equisetum scirpoides* (non-mycorrhizal), the deciduous dwarf shrubs *Betula nana* (with ectomycorrhiza), *Vaccinium uliginosum* (with ericoid mycorrhiza) and *Arctostaphylos alpina* (with arbutoid mycorrhiza), and the evergreen dwarf shrubs *Andromeda polifolia* and *Empetrum hermaphroditum* (both with

ericoid mycorrhiza). Also sporadically present were the herb *Tofieldia pusilla* (non-mycorrhizal), the evergreen dwarf shrub *Rhododendron lapponicum* (ericoid mycorrhiza) and a few other herbs and dwarf shrub species. Three species (*Carex vaginata*, *Empetrum hermaphroditum* and *Vaccinium uliginosum*) were additionally sampled after five days. Mosses and lichens were not analyzed as they rely mainly on N from deposition and associative N_2-fixation.

Additional samples for analysis of ^{15}N natural abundance in plant leaves and soil were collected in unlabelled plots on July 21. The data on ^{15}N natural abundance and details on mycorrhizal status of the plant species are published in Michelsen et al. (1998). Total and green aboveground plant biomass (n = 8) was determined by complete harvest of 20×20 cm plots. Fine and coarse root biomasses of all species were determined for each 2 cm downwards in the soil profile (n = 10).

All plant samples were dried at 80°C and crushed with a mill or by scissors and mortar. The $^{15}N/^{14}N$ isotope ratio and the N concentration of the samples of each c. 5 mg packed in tin capsules were analyzed in an elemental analyzer coupled to an isotope ratio mass spectrometer (EA-IRMS).

Soil samples from the labeled plots were sifted through a 2 mm sieve and extracted with 0.5 M K_2SO_4. The soil for amino acid analysis was extracted with water added to the intact cores that were not sifted in order to prevent N-leakage from roots. These extracts were analyzed for amino acid content at the Department of Physical Geography and Ecosystems Analysis in Lund using a high pressure liquid chromatography (HPLC) system from Dionex, including electrochemical detection and the AminoPac PA10 analytical column (Jonsson et al. 2007; Ström and Christensen 2007).

The total microbial biomass N (MicN) was estimated by the fumigation-extraction method (Brookes et al. 1985; Joergensen and Mueller 1996). The fresh soil was vacuum-incubated with chloroform for 24 hrs, and extracted with 0.5 M K_2SO_4. This and non-incubated extracted fresh soil was spectrophotometrically analyzed for NH_4^+ (indophenol-blue reaction) with a Hitachi U 2000 spectrophotometer. Samples were also analyzed for NO_3^- with a Tecator Aquatec analyzer. A further chemical digestion with H_2SeO_3, H_2SO_4 and H_2O_2 yielded dissolved total N (DTN), with DON (dissolved organic nitrogen) = DTN - NH_4^+. Total microbial N (MicN) was calculated as DON in the fumigated samples minus DON in the non-fumigated samples, using 0.4 as extractability factor (Jonasson et al. 1996; Michelsen et al. 1999; Schmidt et al. 1999).

For the $^{15}N/^{14}N$ isotope ratio analysis, the NH_4^+ of the solutions was concentrated using the steam distillation process (Bremner and Keeney 1965) with pH kept at 4-5 by addition of 0.025 M H_2SO_4. The dried ammonium sulphate was re-dissolved with deionized water and mixed with 'Ultrodex' (N free; Pharmacia Biotech) in tin capsules to form a gel. The EA-IRMS system consisted of a Europa Roboprep Elemental Analyzer coupled to a Europa Tracermass Isotope Ratio Mass Spectrometer. The dried soil was analyzed with a Eurovector CN analyzer coupled to an Isoprime isotope ratio mass spectrometer. During analysis, the reference gas was calibrated against certified standards from the International Atomic Energy Agency, and plant material calibrated against certified standards was used as working standard.

The ^{15}N enrichment of the plant material is the concentration (μmol ^{15}N $g^{-1}N$) of the added ^{15}N in the nitrogen of the dried plant. The ^{15}N natural abundance of each of the plant species was subtracted from the atomic percentage (Fry 2006). In calculating ^{15}N enrichment of the soil N pools, the NH_4-N-concentration of the fumigated minus the non-fumigated digested samples (for microbial ^{15}N, $Mic^{15}N$) and the NH_4-N-concentration of the non-fumigated

digested samples (for $DT^{15}N$) was the N concentration. The recovery in the soil was calculated as the percentage of total added ^{15}N label per m^2 recovered in the total dissolved N (DTN), total microbial N (MicN) or total soil N pool.

One-way analysis of variance (ANOVA) and Tukey's test for comparison of means were used to test for a) effects of injection depth, N form and species on the ^{15}N enrichment, b) change in fine root biomass at increasing depth, and c) differences in soil N pools in plots injected with different N forms. Additionally two-way ANOVAs were applied to test for effects of species and injected N form on plant ^{15}N enrichment. The effect of time on ^{15}N enrichment in plants was tested with repeated measures one way ANOVA using Wilks lambda for the repeatedly sampled plant material, within subject effects was tested with linear contrast. Data with $P < 0.05$ were regarded as statistically significant, but $P < 0.1$ was also reported. All statistical analysis were done using SAS (SAS Institute Inc. 2003).

Results

Plant biomass and soil solution characteristics

The dominant plant species (Table 1) were associated with mycorrhizal fungi: *Vaccinium* made up 30% of the total aboveground plant biomass, *Arctostaphylos* 19%, *Andromeda* 12%, *Empetrum* 8% and *Rhododendron* 7%. Less abundant were the non-mycorrhizal species: *Carex vaginata* made up 5%, *Carex parallela* 0.5%, *Equisetum* 2% and *Tofieldia* 1%. Mosses made up 11% and lichens 3%. The plants had significantly more fine roots ($P = 0.007$) and coarse roots ($P = 0.006$) in the top 2 cm soil than in the layers below 4 cm depth (Fig. 1). The total aboveground biomass of all vascular plants, mosses and lichens was 618.0 ± 7.2 g DW m^{-2}, i.e. only a third of the total above- plus belowground plant biomass, which made up 1706.8 ± 35.7 g DW m^{-2}. Leaf mass made up more than half of the total aboveground vascular plant biomass.

Concentrations of amino acids in the soil solution along with NH_4^+-N, and NO_3^--N in water extracts of non-sifted soil are listed in Table 2. The total amino-N pool was 296 ± 4.7 µg N m^{-2}, corresponding to 0.018 µg N g^{-1} DW soil or 2.011 µg amino acid g^{-1} DW soil.

Three weeks after addition of the ^{15}N label, K_2SO_4 extractable sifted soil pools were below the detection limit (of 0.001 g N m^{-2}) for NO_3^-, 0.56 ± 0.05 g N m^{-2} for NH_4^+, 2.73 ± 0.23 g N m^{-2} for dissolved organic N (DON), 3.29 ± 0.27 g N m^{-2} for dissolved total N (DTN) and 10.93 ± 0.90 g N m^{-2} for soil microbial N (MicN).

Label ^{15}N distribution in ecosystem pools

Seven to 13 times more ^{15}N was found in the microbial N pool than in the DTN pool three weeks after addition of the label. The ^{15}N recovery of the microbial N pool tended to be significantly affected by injected N form ($P = 0.0595$, 1 cm injection depth; $P = 0.0981$, 5 cm depth, one way ANOVAs) but not by depth (Fig. 2b). The ^{15}N recovery in the microbial N pool was 87% (1 cm) higher in glutamic acid plots than in NH_4^+ plots and 53% (both in 1 cm and 5 cm) higher in glycine than in NH_4^+ plots. The recovery of ^{15}N in the dissolved nitrogen pool ($DT^{15}N$) was similar for the three N forms at 1 cm depth injection (Fig. 2c). However, with injection at 5 cm depth, there was a significantly ($P = 0.0450$, one way ANOVA) higher concentration of $DT^{15}N$ in plots labeled with ^{15}N glycine than with $^{15}NH_4^+$ (Fig. 2c).

The recovery of ^{15}N in the total soil (i.e. including microorganisms and dissolved N) was 34 - 70 % of the total injected amount (Fig. 2d), and highest in glutamic acid plots. Furthermore, at this time, the effect of N form on

197 total plant leaf ^{15}N recovery was significant (P = 0.0220, 1 cm and P = 0.0012, 5 cm), with the ^{15}N recovery from the
198 NH_4^+ injection 279% (1 cm) higher than from the glutamic acid injection, and 76% (1 cm) and 187% (5 cm) higher
199 than from the glycine injection (Fig. 2a).
200
201 Nitrogen ^{15}N uptake in plants
202 Both three days and 21 days after the injections of label, significant effects of added N form were found in plants (3
203 days: P = 0.0001; 1 cm and P = 0.0305; 5 cm and 21 days: P < 0.0001; 1 cm and P = 0.0003; 5 cm one-way ANOVA)
204 (Fig. 3a and b). Plants had a higher uptake of ^{15}N from NH_4^+ than from the amino acid sources across species at both
205 injection depths. After three days *Empetrum*, *Vaccinium* and *Equisetum* had acquired significantly more ^{15}N from the
206 added NH_4^+ source than from the glutamic acid source, and *Andromeda* had acquired significantly more ^{15}N from the
207 added NH_4^+ source than from both the amino acid sources in the plots with label injected at 1 cm (Fig. 3a). With the
208 label injected at 5 cm (i.e. with no glutamic acid application), *Andromeda* and *Tofieldia* acquired significantly more
209 ^{15}N from the added NH_4^+ source than from the glycine source (data not shown).
210 At 21 days after label injection *Andromeda* and *Carex parallela* had acquired more N from the NH_4^+ source
211 than from glutamic acid, and *Equisetum* and *Carex vaginata* had acquired significantly more N from the NH_4^+ source
212 than from both the amino acids, when label was injected in 1 cm depth (Fig. 3b). *Andromeda*, *Carex vaginata* and
213 *Betula* had acquired significantly more N from the NH_4^+ source than from glycine, when label was injected in 5 cm
214 depth (data not shown).
215 The effect of plant species on ^{15}N uptake was significant both at three days after injection (P = 0.0379; 1 cm
216 and P = 0.0055; 5 cm, one-way ANOVA) and 21 days after injection (P < 0.0001; 1 cm and P = 0.0143; 5cm). The
217 significant effects of N form and species after three days (N form: P = 0.0168, species: P < 0.0001 two-way ANOVA)
218 and after 21 days (N form: P < 0.0001, species P < 0.0001) at 1 cm depth (Fig 3a and b), persisted throughout all the
219 samplings.
220 The mycorrhizal status had significant effect on ^{15}N allocation to aboveground plant tissue three days after
221 labeling for all N forms at both depths, with more ^{15}N uptake in the non-mycorrhizal species than in the mycorrhizal
222 species (1 cm depth injection: P = 0.0098 for $^{15}NH_4$, P = 0.0008 ^{15}N for glycine, P = 0.0360 ^{15}N for glutamic acid; 5
223 cm depth injection: P = 0.0107 for $^{15}NH_4$, P = 0.0009 ^{15}N for glycine). By contrast, 21 days after injection, the
224 mycorrhizal species had significantly larger uptake of ^{15}N in glutamic acid plots (P = 0.0007) and in glycine plots (P =
225 0.0051, 1 cm and P = 0.0478, 5 cm), but there was no effect of mycorrhizal status on ^{15}N uptake in $^{15}NH_4$ plots (P =
226 0.6266, 1 cm and P = 0.1801, 5 cm).
227 The three species analyzed at all three sampling times after injection in 1 cm depth, increased ^{15}N enrichment
228 from the three N form additions significantly through time (*Carex vaginata*: P < 0.0001 $^{15}NH_4^+$, P = 0.0002 gly;
229 *Empetrum*: P < 0.0016 gly; *Vaccinium* P = 0.0002 $^{15}NH_4^+$, P < 0.0135 gly, P = 0.0177 glu; analyzed with repeated
230 measurements ANOVA) (Fig. 4).
231 Three days after label injection, the uptake of $^{15}NH_4^+$ from the 5 cm depth injection was significantly lower
232 than the uptake from 1 cm depth for *Andromeda*, *Empetrum,* and *Vaccinium* (P = 0.0489, P = 0.0014, and P =
233 0.0083), and tended to be so for *Equisetum* (P = 0.0813) (Fig. 5a). The ^{15}N uptake in plots with glycine injected in 5
234 cm depth was slightly lower for *Vaccinium* (P = 0.0037) and tended to be so for *Equisetum* (P = 0.0847) (Fig. 5b).

Discussion

Soil solution characteristics

The measured total amount of free amino acids in the soil water was small. Although the knowledge of pools and turn-over times of amino acids in different soils is limited (Jones et al. 2005a; Weintraub and Schimel 2005; Kielland et al. 2007), the concentrations found were low compared to several earlier reports (Abuarghub and Read 1988a; Abuarghub and Read 1988b; Kielland 1995; Finzi and Berthrong 2005; Sorensen et al. 2008). The ratio of total amino acids to inorganic N was 1:27, and the amino acid concentration was one and two orders of magnitude lower than at a nearby heath (Sorensen et al. 2008), and at a non acidic site in Alaska where pH and the vegetation was very similar (Nordin et al. 2004). This difference was probably due to the methods of processing the soil samples; water extracts were used from non-sifted soil to prevent unwanted N-leak from any damaged 'sifted' roots. The difference could also be due to the different analytical methods as, e.g., use of water as extractant, or of NH_4OAC or KCl (Abuarghub and Read 1988a; Finzi and Berthrong 2005) or the use of HPLC (here and Abuarghub & Read 1988b) vs. the ninhydrin-reaction (Abuarghub and Read 1988a; Finzi and Berthrong 2005; Kielland et al. 2007).

Acquisition of organic or inorganic nitrogen

Soil microorganisms and plants differ in rates of acquisition of the wide range of N-containing inorganic and organic compounds available in soil water. As in other *in situ* studies (Schimel and Chapin 1996; Grogan and Jonasson 2003; Nordin et al. 2004) a rapid uptake of ^{15}N by microbes was observed through the first three weeks after labeling with a recovery of 24 - 47% of the added amounts. This high recovery supports the hypothesis of higher microbial than plant uptake a few weeks after label addition, with the plant leaf ^{15}N recovery being less than 2.5%. Hence, in the short term, the microbes are superior to plants in their competition for N, irrespective of added N form.

Our hypothesis that plants and microorganisms would differ in uptake of the added ^{15}N forms was also supported by the study. The plants had consistently higher uptake of ^{15}N from the $^{15}NH_4^+$ source than from the ^{15}N amino acids, while the ^{15}N enrichment in the soil microorganisms and in DTN was lowest in the $^{15}NH_4^+$ labeled plots. The difference in ^{15}N uptake between plants and microorganisms suggests that soil microbes with their large uptake also control the partitioning of pulse-released nitrogen between microorganisms and plants: relatively more $^{15}NH_4^+$, and relatively less amino N is left for plant acquisition. The high microbial $^{15}NH_4^+$ uptake potential in this experiment suggests that microbial immobilization of NH_4^+ can reduce plant N acquisition (Schmidt et al. 1999), although the effect may be more pronounced in pulse releases following dry-rewet or freeze-thaw incidents (occurring during shoulder and growing season, (Konestabo et al. 2007), than in a situation with more gradual release of N from decaying organic matter.

In our study, larger plant acquisition of inorganic N than of organic N was generally observed across all ten species. This agrees with previous studies demonstrating larger uptake of inorganic nitrogen than of N from amino acid sources by plants (Kielland 1994; Lipson et al. 1999; Falkengren-Grerup et al. 2000; Miller and Bowman 2003; Bennett and Prescott 2004; Månsson 2005), although there is no proof of intact uptake of the amino acids in our experiment. In assays where organisms are given a choice between N forms in a mixed solution, the question of N

form preference may be more truly addressed and reveal both preference and lack of preference (Schimel and Chapin 1996; Bardgett et al. 2003; Nordin et al. 2004; Kielland et al. 2006).

The higher microbial uptake in this experiment of N from the added glutamic acid (with a C to N ratio of 5) than of N from glycine (with a C to N ratio of 2), furthermore agrees with earlier suggestions of microbial preference for the amino acids with highest C to N ratio, perhaps due to microbial C limitation (Lipson et al. 1999; Michelsen et al. 1999; Schmidt et al. 2000; Nordin et al. 2004). The cellular transmembrane uptake of glutamic acid (glutamate) may be facilitated since glutamic acid enters into the glutamine synthetase-glutamate synthase pathway, whereas acquired ammonium first must be coupled to α-ketogluterate to form glutamate (Paul and Clark 1996). The further metabolic pathway of the carbon from the acquired amino acid compound is not investigated in this study, but in a ^{14}C labeling experiment with a mixture of 15 amino acids as much as 25% of the carbon was used for respiration and the remainder incorporated in microbial biomass (Jones and Kielland 2002).

It appears that N form preference differs among ecosystem types, or perhaps that the differences are caused by methodological differences such as label concentration, differences in pool dilution or plant species and microbial community composition (Vinolas et al. 2001; Jones et al. 2005b). Comparing pools and fluxes of different nitrogen compounds in one experiment has unavoidable difficulties. The ^{15}N label of the investigated N forms was added with the same amount of N per m^2, but when compared to the ambient pool sizes of NH_4^+, glycine and glutamic acid, the dilution of the ^{15}N label differed between N forms: The amount of $^{15}NH_4^+$ label approached a fourth of the NH_4^+ in the soil solution, while the ^{15}N glycine and glutamic acid label increased the soil solution concentrations more than thousand fold. Even so, the soil solution concentrations of amino acids and inorganic N indicate that these compounds are naturally available as substrate or nutrients. Furthermore, a high concentration of one compound (e.g. NH_4^+) does not necessarily represent a concomitant high flux of this compound, and a measured low concentration of amino acids may 'hide' a high flux of these compounds (Weintraub and Schimel 2005). Half-lives of amino acids of less than 24 hrs in sub-arctic and arctic soils have been reported (Jones and Kielland 2002; Finzi and Berthrong 2005), and the large uptake of amino acids by microorganisms in our experiment indicates that the flux into microorganisms is potentially large. ^{15}N-nitrate was not included in our study, because most of the plant species at the site do not show nitrate reductase activity, despite the occasional presence of low NO_3^- concentration in the soil (Michelsen et al. 1996).

The ^{15}N-recovery in the total soil, comprising adsorbed, dissolved and microbially immobilized ^{15}N was high (34% -70%), but as plant ^{15}N uptake only comprised a minor part of the recovered ^{15}N, downwards leaching of ^{15}N-label, like of pulse released N after freeze-thaw or dry-rewet through the soil horizon, is likely.

N acquisition patterns in plants

The ^{15}N enrichment in plants three days after addition of the labeled compounds suggests a significant ability to utilize the added compounds, although the extent to which the ^{15}N was acquired in form of the originally added compound or on a decomposed/mineralized form (^{15}N-glycine, $^{15}NH_4$, $^{15}NO_3$) of the original, can not be quantified. The consistent, although not always significantly smaller ^{15}N uptake from 5 than 1 cm depth by all species, agrees with larger fine root biomass in the top soil layers. The small difference between species suggests, that all species on this species-rich heath mainly exploited the uppermost soil layer for N. However, *Empetrum* and *Vaccinium* seemed to rely more

309 strongly on N uptake from the surface soil, irrespective of N form. This emphasizes the relevance of carefully
310 choosing depth of injection in labeling experiments when investigating interspecific competition in plant communities.
311 An indication (though not significant) of differences in the downwards diffusion of the N forms was also
312 demonstrated, with the relatively higher plant uptake from 5 than 1 cm injection depth in glycine than in NH_4^+
313 injections. This may indicate that the glycine ^{15}N label percolates faster than the $^{15}NH_4^+$ label down through the top
314 soil but not through the deeper soil, possibly depending on different adsorption potentials in different soil layers.
315 Our hypothesis of higher ^{15}N-uptake from the added organic sources by plant species with mycorrhizal
316 associations than by non-mycorrhizal plant species was only partially supported by the study. At first, after three days,
317 the ^{15}N concentration was largest in non-mycorrhizal species but after 21 days the mycorrhizal species had acquired
318 more ^{15}N from the amino acid sources than had the non-mycorrhizals. The delay in the uptake of ^{15}N by the
319 mycorrhizal species could perhaps be explained by dependence on transcription induction of membrane amino acid
320 transporters in the cell membrane of the mycorrhizal fungi (Chalot et al. 2002), eventually giving a larger ^{15}N amino
321 acid uptake. However, as amino acids are constantly available in the soil solution, this seems less likely. The differing
322 uptake patterns of the plant functional types agree with earlier observations of high spring-time uptake rate and
323 allocation to leaves in graminoids, and slower uptake and allocation in the woody ericoid species (Andresen and
324 Michelsen 2005).
325 Species of the Ericales (*Andromeda*, *Arctostaphylos*, *Empetrum*, *Rhododendron* and *Vaccinium*) have a dense
326 network of thin, hair-like roots giving the plant a large surface for N uptake, while the monocotyledonous *Carex* spp.
327 and *Tofieldia* have thicker roots in patches. Hence, species differences in root form and rooting pattern may also cause
328 variation in access to the label (Xu et al. 2006). However, the monocots with presumed lower root surface actually had
329 higher uptake potential than dwarf shrubs of Ericales (*Empetrum*, *Rhododendron*, *Vaccinium*) at three but not 21 days
330 after labeling.
331 The fast N uptake by monocots versus a slower but larger N uptake by stress-tolerant dwarf shrub species
332 with lower N demand (Michelsen et al. 1999) suggests that the presence of mycorrhizae, giving the plant extended
333 surface for N uptake, is of more value in a longer term acquisition strategy. Furthermore the accumulated ^{15}N recovery
334 in the plants 21 days after injection (Fig. 2a) demonstrates that most of the plant acquired ^{15}N on this site is found in
335 leaves of the Ericales species, which reflects their biomass dominance (McKane et al. 2002).
336

337 ## *Conclusions*

338 In accordance with the hypotheses, the soil microbes took up ^{15}N most efficiently and with higher uptake from the
339 added amino acid sources than from the NH_4^+ source. The ^{15}N uptake by plants was much higher from the NH_4^+
340 source than from the amino acid source, controlled by the microbial uptake. The non-mycorrhizal plant species
341 showed a fast uptake from the pulse addition of the ^{15}N sources, while the mycorrhizal plant species had delayed but
342 eventually larger ^{15}N uptake from the amino acid sources than the non-mycorrhizal plants, and similar uptake from the
343 $^{15}NH_4^+$ source. All plant species in this species-diverse heath preferentially exploited the uppermost soil layer, and
344 hence competed spatially.
345

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351

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Fig. 1 Soil profile coarse root (grey) and fine root (hatched) biomass (g DW m^{-2}) in the soil profile. Bars with the same letters are not significantly different with Tukey's test; $P < 0.05$.

Fig. 2 ^{15}N recovery in **a)** in leaves of plant species: Cp *Carex parallela*, Cv *Carex vaginata*, Eq *Equisetum scirpoides,* An *Andromeda polifolia*, Em *Empetrum hermaphroditum*, Va *Vaccinium uliginosum,* Rh *Rhododendron lapponicum,* Ar *Arctostaphylos alpina* and Be *Betula nana* **b)** in total microbial biomass, **c)** in dissolved total N and **d)** in total dried soil 21 days after labeling with $^{15}NH_4^+$, ^{15}N -glycine or ^{15}N -glutamic acid in 1 cm or 5 cm depth (mean ± SE). Effect of N form for each depth was analyzed with one-way ANOVA; * $P < 0.05$. Within injection depth columns with the same letters or no letters are not significantly different with Tukey's test; $P < 0.05$; letters in parentheses when $P < 0.1$.

Fig. 3 a) ^{15}N enrichment in plants three days after labeling and **b)** 21 days after labeling in 1 cm depth with $^{15}NH_4^+$, -glycine or -glutamic acid (mean ± SE). The species are: *Tofieldia pusilla*, *Carex parallela*, *Carex vaginata*, *Equisetum scirpoides, Andromeda polifolia*, *Empetrum hermaphroditum*, *Vaccinium uliginosum,* and *Arctostaphylos alpina*. Significant effect of species and N form was analyzed with two-way ANOVA; * $P < 0.05$; *** $P < 0.001$. Within species columns with the same letters or no letters are not significantly different with Tukey's test; $P < 0.05$. *Tofieldia* was not tested after three days due to low replication.

Fig. 4 ^{15}N-enrichment 3, 5 and 21 days after labeling in **a)** *Carex vaginata*, **b)** *Empetrum hermaphroditum*, and **c)** *Vaccinium uliginosum*, (mean ± SE). The effect of time was analyzed with repeated measurements ANOVA. Columns with the same letters or without letters are not significantly different as tested with linear contrasts; $P < 0.05$.

Fig. 5 Percentage ^{15}N uptake in plant leaves three days after labeling, from 5 cm depth injection relative to uptake from 1 cm depth injection from **a)** $^{15}NH_4^+$ labeled plots and **b)** ^{15}N-glycine labeled plots. † $P < 0.1$; * $P < 0.05$, ** $P < 0.01$ for difference in uptake from the two depths, one-way ANOVA. Only five species had enough replicates for all combinations of injection depth and N form to allow comparison.

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Abstract:

This manuscript provides new information of competition between soil microorganisms and plants of a Danish temperate heathland ecosystem. With use of the stable isotopes ^{15}N and ^{13}C used in an *in situ* injection, the uptake of ammonium and the amino acids glycine, glutamic acid and phenylalanine is quantified in plants and soil microorganisms. Overall the plant:microbial ^{15}N recovery ratio was 1:12, hence, the soil microorganisms were superior to plants in the short term competition for the nitrogen pulse. Soil microorganisms showed significant uptake of intact amino acid molecules. The plants *Calluna vulgaris* and *Deschampsia flexuosa* showed preference of the inorganic nitrogen source where as microorganisms showed no preference. Ecosystem biomass and C and N pools of the plant species and soil microorganisms are reported. Seasonal variations of the field site soil amino acids, plant root biomass, microbial biomass and ammonium is also reported.

Free amino acid and ammonium uptake in temperate heathland vegetation and soil microorganisms under influence of enhanced soil tannic acid

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Abstract

In heath soil, free amino acids can serve as substrates for soil microorganisms and are acquired as nutrients directly by plants. Phenolic compounds (tannins) in the soil inhibit microbial activity and complex bind labile nutrients such as amino acids.

In this experiment we increased the soil tannic acid concentration and investigated the uptake of added amino acids (glycine, glutamic acid and phenylalanine) and ammonium by soil microorganisms and heath plants: *Calluna vulgaris* and grasses (mainly *Deschampsia flexuosa*). ^{15}N ammonium and fully $^{15}N^{13}C$-labeled amino acids and tannic acid were injected into the soil. Uptake of intact amino acids was seen in sample ^{13}C:^{15}N ratios one day after labelling. Uptake of ammonium and all amino acids was highest in the microbial biomass, with a ^{15}N label recovery of 26 - 53% after one day and with no significant preference of nitrogen form. The vascular plant species showed significant preference for ammonium and had a ^{15}N label recovery of only 0.4 - 3.9 %. Translocation of the acquired nitrogen was observed through the plant fractions. Tannic acid addition reduced both dissolved organic N concentration and ^{15}N recovery in the total dissolved soil N pool, and furthermore reduced the ^{15}N recovery of some of the N forms in *Calluna*.

Overall, the plant : microbial ^{15}N recovery ratio was 1:12, hence, the soil microorganisms were superior to plants in the short term competition for the added nitrogen pulse. As both plants and microorganisms show capability for uptake of ammonium and amino acids from the same pools, rapid fluxes, high uptake rates and alternating mineralization and immobilization of nutrients in plants and microbes are important elements of nutrient cycling in terrestrial ecosystems.

Key words: amino acid, competition, nitrogen, translocation, heathland.

1. Introduction

Soil microorganisms and plants acquire nitrogen from both inorganic (NO_3^- and NH_4^+) and organic sources (amino acids), and acquire intact amino acids (Nordin and Näsholm 1997; Näsholm et al., 1998; Persson and Näsholm 2001; Hofmockel et al., 2007). The free amino acids in the soil pore water origin partly from rhizo deposition (Lesuffleur et al. 2007; Ström and Christensen 2007) and partly as leachates from decomposing organic matter. In tundra soils protease activity controls protein breakdown and release of amino acids (Weintraub and Schimel 2005a; Weintraub and Schimel 2005b), and in boreal forest soil the half-life of free amino acids is short (Kielland et al. 2007), due to fast uptake of the mineralized or intact amino acids by competing plants and soil microorganisms.

The leaves and roots of plants from the Ericales have high concentrations of phenols (condensed and hydrolysable tannins) (Jalal et al. 1982; Frutos et al. 2002; Hansen et al. 2006). Heathland soil consequently has high concentrations of lignin-derived phenolic compounds (Bending and Read 1996; Kraus et al. 2003). In soil, phenols react with amino acids to form humate, followed by complex binding to peptides in the chemical process of humification (Paul and Clark 1996). In forest ecosystems this may control nutrient dynamics through delayed decomposition of soil organic matter (Northup et al. 1998; Kraus et al. 2003) through chemical complex-binding of tannins and labile nutrients (Halvorson and Gonzales 2008). Decomposing soil microorganisms may respond to high soil concentrations of tannins with inhibited growth, but in some cases with decomposition of tannic acid (Kraus et al. 2003). For instance, the protease activity of *Hymenoscyphus ericae*, an ericoid mycorrhizal forming fungi with the heathland dwarf shrub *Calluna vulgaris*, is unaffected by tannic acid (Bending and Read 1996).

63 In this fashion, plant production of phenolics and subsequently the chemical humification in the
64 soil and protease production by the ericoid mycorrhizal fungi, may control nitrogen cycling at
65 heathlands (Bending and Read 1996; Kraus et al. 2003).

66 Partitioning of nitrogen from the soil pools between plants and microorganisms has been
67 estimated with biomass and growth measurements in e.g. fertilization experiments (Jonasson et
68 al. 1996; Michelsen et al. 1999; Schmidt et al. 1999) and more recently also with tracer studies
69 using the stable ^{15}N isotope (Nordin et al. 2004; Sorensen et al. 2007; Harrison et al. 2008).
70 Addition of nitrogen to heath ecosystems may result in larger microbial biomass (Schmidt et al.
71 1999) and in the long term cause changes in plant species dominance (Aerts 1990; Jonasson et
72 al. 1999). By using ^{15}N to trace the nutrient flow through the pools in the soil-microorganism-
73 plant system, competition for very small, non-fertilizing pulses of nitrogen can be investigated.

74 In this experiment, a comparison is made of uptake of ammonium-N and amino acid-N
75 in the form of either glycine (aliphatic amino acid, C:N ratio is 2), glutamic acid (acidic amino
76 acid, C:N ratio is 5) or phenylalanine (aromatic amino acid, C:N ratio is 9) by soil
77 microorganisms and heathland plants, *viz.* grasses (mainly *Deschampsia flexuosa*), the evergreen
78 dwarf shrub *Calluna vulgaris*, and mosses. The ammonium was labelled with ^{15}N and the amino
79 acids with ^{15}N and ^{13}C. These were added to the soil in very low concentration to trace the N and
80 C fluxes and to estimate the amount of amino acids acquired in intact form. The effect of tannic
81 acid addition to the soil on nitrogen uptake and soil chemistry was also investigated.
82 It was hypothesized:

83 - That both microorganisms and plants would be able to absorb N in both the added
84 inorganic and organic forms.

- That the dominant grasses and *Calluna vulgaris* would take up lower amounts of added nitrogen than soil microorganisms following labelling.
- that the rate of translocation of the absorbed ^{15}N shortly after labelling would be observed as gradually decreasing concentration from fine root to leaf tissue.
- That addition of tannic acid would reduce the amount of extractable DOC and DON.
- That addition of tannic acid would reduce the available and extractable amount of the added, labile, amino acids and lead to smaller uptake of ^{15}N by plants.

2. Materials and methods

The experiment took place at the site of the CLIMAITE experiment (Mikkelsen et al. 2007) at Brandbjerg (55º53'N 11º58'E) c. 50 km NW of Copenhagen, Denmark. The site was a managed, dry, temperate heath on a hilly nutrient-poor sandy deposit, with an organic layer of c. 5 cm depth and a pH of about 5. The vegetation was dominated by *Calluna vulgaris*, *Deschampsia flexuosa* and *Festuca ovina* accompanied by heathland mosses and herbs. The average precipitation per year was about 600 mm and the average temperature was 8° C.

2.1 In situ injection

Fifty four plots of 20×20 cm were chosen to contain an equal amount of *Calluna vulgaris* (evergreen dwarf shrub) and grasses (mainly *Deschampsia flexuosa* but also *Festuca ovina*). Six of the plots were kept unlabeled for analysis of ^{15}N and ^{13}C natural abundance. On May 18 2005, 24 labelled plots were initially amended with tannic acid ($C_{76}H_{52}O_{46}$; $\delta^{15}N$ -2.13; $\delta^{13}C$ -25.04) each plot receiving 1 dl of re-demineralised water with 0.88 g tannic acid equal to 22 g of tannic acid added pr. m^2. To each of the 48 plots a nutrient solution was amended, weighed out

with the same amounts of N from ammonium, glycine, glutamic acid and phenylalanine. For 12 plots the ammonium was labelled with ^{15}N (99%^{15}N ammonium chloride, NH_4Cl) each plot receiving 1 dl of re-demineralised water with 0.007 g NH_4Cl. For other 12 plots the glycine was labelled with ^{15}N and ^{13}C (U-$^{13}C_2$, 98%; ^{15}N 98% glycine, H_2NCH_2COOH) each plot receiving 1 dl of re-demineralised water with 0.001 g glycine. For other 12 plots the glutamic acid was labelled with ^{15}N and ^{13}C (U-$^{13}C_5$, 98%; ^{15}N 98% L-glutamic acid $HOOC(CH_2)_2CH(NH_2)COOH$) each plot receiving 1 dl of re-demineralised water with 0.002 g glutamic acid. Finally, for other 12 plots, the phenylalanine was labelled with ^{15}N and ^{13}C (U-$^{13}C_9$, 98%; ^{15}N 98% L-phenylalanine $C_6H_5CH_2CH(NH_2)COOH$) each plot receiving 1 dl of re-demineralised water with 0.020 g phenylalanine. Hence, the relative amount of added ^{15}N was 10 : 1 : 1 : 10 for Phe, Glu, Gly and Amm, and likewise 90 : 5 : 2 : 0 for ^{13}C, in the four different labelling solutions. pH of the solutions was adjusted from 3.7 with NaOH to 4.7 as measured in soil water solution. The total amount of added N was 0.2 $gN \cdot m^{-2}$. This gave six replicate plots to follow uptake of ammonium from the nutrient solution and 6 replicate plots to follow uptake when tannic acid had been supplied, and likewise for glycine, glutamic acid and phenylalanine. The label was injected into the soil just below the soil surface with a syringe at 20 evenly distributed points within the 20×20 cm plots.

2.2 Sampling from the labelled plots

One day after labelling, above ground (down to soil surface) vegetation was sampled and sorted into shoots of *Calluna*, *Deschampsia* (including leaf meristem) and mosses (mixture of species). The samples were kept cold on ice until they were freeze dried and analyzed for ^{15}N and ^{13}C isotopic enrichment. Additionally, one day after labelling, soil cores were sampled from the soil surface (including the litter layer) and down to 5 cm depth. Three soil cores (diam. 4.5 cm) were

130 taken from each plot, mixed to a composite sample and immediately sorted into roots and soil.
131 All plant material was washed with 0.5 mM $CaCl_2$, frozen and freeze dried. A subsample of the
132 fresh soil from each plot was extracted with re-demineralised water (1:5) on a shaker for 1 hr.
133 and another set of subsamples was vacuum-incubated with chloroform for 24 hrs to release
134 microbial C and N (Brookes et al. 1985; Joergensen and Mueller 1996) before extraction with
135 water as above. A third subsample of the sorted and sifted soil was freeze dried and used for
136 estimating soil water content. One and a half year after labelling, additional soil samples for
137 measurements of longer-term distribution of the labels were taken from the plots in three depths
138 of 0-5 cm, 5-10 cm and 10-15 cm.

139 One week after labelling, all aboveground plant material was sampled from the plots in
140 order to obtain plant biomass estimates and ^{15}N and ^{13}C natural abundances from the six
141 unlabelled plots. The *Calluna* material was sorted into green shoots, coarse, non-green branches,
142 coarse roots and fine roots (< 0.5 mm) and the grasses were sorted into leaves, coarse and fine
143 roots (< 1 mm). Mosses and aboveground litter of mainly grasses but also *Calluna* constituted
144 additional fractions.

145 Soil samples for analysis of seasonal variation of the masses of amino acids, microbial
146 biomass C and N, soil extractable NH_4^+, NO_3^-, DON, DOC and fine roots of *Calluna* and grasses
147 were collected under a mixed graminoid and *Calluna* vegetation in plots adjacent to the labelled
148 plots on February 21st, April 4th, May 11th, June 28th, July 27th, August 23rd 2005, and January
149 16th 2006). After washing, the roots were sorted into fine roots (<0.1 mm) of *Calluna* and grass
150 roots smaller than 0.5 mm in diameter. Soil for analysis of microbial biomass C and N, and soil
151 extractable NH_4^+, NO_3^-, DON, DOC was treated as above. Also, a subsample was used for

152 analyses of amino acids after extraction with re-demineralised water (1:2) and centrifugation at
153 10000 rpm (11951g) for 15 minutes.

154 ## 2.3 Chemical and isotopic analysis

155 The soil extracts were spectrophotometrically analyzed for NH_4^+ (indophenol-blue reaction)
156 with a Hitachi U 2010 spectrophotometer and for NO_3^- with a Tecator FIAstar analyzer. Part of
157 the extract was digested with H_2SeO_3, H_2SO_4 and H_2O_2 and analyzed as above to yield total
158 dissolved N (TDN), with DON (dissolved organic nitrogen) = TDN - NH_4^+. Total microbial N
159 (MicN) was calculated as TDN in the fumigated samples minus TDN in the non-fumigated
160 samples, using 0.4 as the extractability factor (Jonasson et al. 1996; Michelsen et al. 1999;
161 Schmidt et al. 1999). Another part of the extract was analyzed for organic carbon (DOC) with a
162 Shimadzu TOC 5000A analyzer. Total microbial C (MicC) was calculated as DOC in the
163 fumigated samples minus DOC in the non-fumigated samples, using 0.45 as the extractability
164 factor (Schmidt et al. 2000).

165 The centrifuged soil extracts were analyzed for amino acid content on a Dionex HPLC
166 system (column: AminoPac PA10) following the method of Ström and Christensen 2007
167 (Jonsson et al. 2007; Ström and Christensen 2007).

168 Milled leaves of *Deschampsia* and leaves and fine roots of *Calluna*, collected on August
169 22nd (leaves) and September 9th 2007 (roots), were extracted with methanol and analyzed for
170 condensed tannins by the vanillin method with catechin as standard, using a Hitachi U 2010
171 spectrophotometer (Burns, 1971).

172 For the $^{15}N/^{14}N$ and $^{13}C/^{12}C$ isotope ratio analysis of the fumigated and non fumigated
173 soil extracts, the extracts were freeze-dried in a small bottle containing a quartz filter (Quartz
174 microfibre filters QMA Whatman) and with a small parafilm lid with a small hole. Filters, dried

175 crushed soil and plant material were analyzed for ^{15}N and ^{13}C isotopic enrichment with a
176 Eurovector CN analyzer coupled to an Isoprime isotope ratio mass spectrometer. During
177 analysis, the internal reference gas was calibrated against certified standards from the
178 International Atomic Energy Agency, and plant material calibrated against certified standards
179 was used as working standard.

180 ## 2.4 Calculations and statistics

181 The ^{15}N and ^{13}C enrichments of the plant material and the microbial biomass was assumed to be
182 equal to the concentrations ($\mu mol \cdot g^{-1}$ dry weight, DW) of the added ^{15}N or ^{13}C in the material.
183 The atomic percentage was calculated from $\delta^{15}N$ values (atom% = $(\delta^{15}N +1000)/((\delta^{15}N$
184 $+1000+(1000/0-0.0036765)))$ or ^{13}C values (atom% = $(\delta^{13}C +1000)/((\delta^{13}C +1000+(1000/0-$
185 $0.011180)))$. The measured ^{15}N or ^{13}C natural abundance of the material was then subtracted and
186 this figure was multiplied by the N or C concentration of each sample, giving the ^{15}N or ^{13}C
187 enrichment (Fry, 2006). The ^{15}N recovery was calculated as the percentage of total added ^{15}N
188 label per m^2 recovered in the total dissolved N (TDN), total microbial N (MicN), total soil N
189 pool and in the plant biomass pr m^2.

190 One-way analysis of variance (ANOVA) and Tukey's test for comparison of means were
191 used to test for difference in ^{15}N enrichment between species, change in fine root biomass at
192 increasing depth, differences in soil N pools in plots injected with different N forms, and effects
193 of time. Two-way ANOVAs were applied to test for effects of species and injected N form, and
194 injected N form and addition of tannic acid. Interactions between main effects were included
195 and are reported when significant. Homogeneity of variances was tested with Levene's test prior
196 to the analysis of variances. Data with $P \leq 0.05$ are regarded as statistically significant, but

197 effects at $P \leq 0.1$ are also reported. All statistical analyses were done using SAS (SAS Institute
198 Inc. 2003)

199 *3. Results*

200 3.1 Soil properties and seasonal variations

201 Ammonium was the dominant inorganic N form in the soil (Table 1). The concentration of
202 dissolved organic N (DON) was eight-fold higher than the concentration of NH_4^+-N and
203 microbial N was 17-fold higher than DON. The total amount of free amino acid N corresponded
204 to 0.4 % of the measured dissolved organic N. The microbial C:N ratio was 10, indicating a
205 mixed microbial community of bacteria and fungi (Jensen et al. 2003). Mean organic matter
206 percentage of the soil (SOM) was 11.1 ± 0.6 % (S.E.). The soil pools and concentrations were
207 not significantly affected by the addition of the small amount of isotope label (two-way
208 ANOVA). Addition of tannic acid had a significant effect on extractable DOC (P=0.0166) and a
209 tendency towards an effect on extractable DON (P=0.0666, one way ANOVA), with more DOC
210 and less DON in plots with tannic acid addition (data not shown).
211 The seasonal variations of most of the single amino acids, the NH_4^+ concentration and
212 the microbial biomass (Figure 1 and 2) were significant. The concentration of amino acids was
213 generally highest in August, intermediate in May and lowest in June (Figure 1). For NH_4^+-N
214 (Figure 2) the concentration was highest in March, decreased (P<0.0001) to a minimum in
215 August and had increased significantly by January the subsequent year. The microbial N mass
216 (Figure 2) increased (P<0.0001) from March to May, decreased from May to August and tended
217 to have increased by January. Nitrate decreased (P=0.0009) from March to May.

3.2 Plant biomass and chemistry

The vegetation was dominated by *Calluna* with an average total above- plus belowground biomass of 715 $g \cdot m^{-2}$ and by grasses with an average total above- plus belowground biomass of 460 $g \cdot m^{-2}$. The average total plant biomass was 1200 $g \cdot m^{-2}$ (Table 3, measured in May). The fine root biomass of grasses was three- to 20-fold higher than the fine root mass of *Calluna* (Figure 3) in 0-5 cm depth with a significant four-fold increase from March to September followed by a significant decrease to January, while the fine root biomasses of *Calluna* did not vary significantly through seasons.

The largest ecosystem nitrogen pool (Table 2) was in *Calluna* with 7.0 $g \cdot m^{-2}$ while the grasses and mosses contained 5.2 and 1.2 g $N \cdot m^{-2}$, respectively, adding up to a total plant pool of 13.4 g $N \cdot m^{-2}$. The microbial biomass contained 0.8 g $N \cdot m^{-2}$ (Table 3).

The concentration of condensed tannins in leaves of *Deschampsia* was below the detection limit, while the concentration in leaves and fine roots of *Calluna* was 39 ± 3 $mg \cdot g^{-1}$ DW and 73 ± 5 $mg \cdot g^{-1}$ DW, respectively.

3.3 ^{15}N label recovery

One day after labelling, 45 - 89 % of the ^{15}N label was recovered in the upper 5 cm soil (Table 3) of which labile pools such as the microbial biomass and the TDN pool contained 26 - 53 % and 0.1 - 1.3 % respectively. Hence, the difference of 19 - 35 % of the added label recovered in the total soil and the amount recovered in these labile pools presumably represents ^{15}N adsorbed to the soil particles. After 1.5 yr, less, 9 - 53 %, of the ^{15}N label was recovered in the upper 5 cm and ^{15}N recovery decreased with soil depth (data not presented).

239 ^{15}N label recovery was much higher in the soil microbial ^{15}N pool than in *Calluna* and
240 the grasses, with 0.4 - 3.9 % and 1.2 - 3.9 %, respectively. The recovery was very low in mosses
241 (0.03% at most) (Table 3).

242 For *Calluna*, there was a significant effect of N-form and a significant interaction of N-
243 form and tannic acid (Table 3), with the highest ^{15}N recovery from the ammonium and the
244 phenylalanine labelling. In the grasses, there was a tendency towards an effect of N-form and a
245 higher ^{15}N recovery from the ammonium labelling (Table 3). N-form had no significant effects
246 on ^{15}N recovery for mosses, microbial N, total dissolved N and total soil N (Table 3).

247 There was a significantly (P=0.0292) larger ^{15}N recovery in grasses than in *Calluna* in
248 plots labelled with glycine, and a tendency (P=0.0511) towards this in plots labelled with
249 glutamic acid, despite the higher biomass and unlabeled N pool in *Calluna*.

250 Addition of tannic acid had no significant effect on ^{15}N label recovery in plants or soil
251 microorganisms (Table 3) and no effect on ^{15}N enrichment of the plant fractions (data not
252 shown), but decreased the total dissolved ^{15}N (Table 3).

253 ## 3.4 ^{15}N and ^{13}C enrichments

254 One day after labelling, the ^{15}N concentration in the microbial biomass had increased
255 significantly above the natural abundance in plots with added NH_4^+. Similarly, both the ^{15}N and
256 the ^{13}C concentrations had increased in plots with added, labelled, amino acids, indicating
257 microbial uptake of all compounds (Table 3 and Figure 5). Both the grasses and *Calluna* had
258 absorbed significant amounts of ^{15}N from the added $^{15}NH_4^+$, but, in the amino acid plots, the
259 concomitant increase of both ^{15}N and ^{13}C was significant only in the phenylalanine plots (Table
260 3).

The linear relationships of excess ^{13}C and ^{15}N in soil microorganisms in plots with the labelled amino acids were significant at $P < 0.0001$ in all cases (Figure 4), as was the linear relationship in grasses and *Calluna* in the labelled phenylalanine plots (Figure 5). In contrast, in the plants, there were no significant linear correlations in plots with labelled glycine and glutamic acid, perhaps due to the fact that the amount of ^{15}N added with these acids only was 1/10 of the amount added with phenylalanine.

4. Discussion

The soil had low concentrations of free glycine, glutamic acid and phenylalanine (Abuarghub and Read 1988; Kielland et al. 2006; Sorensen et al. 2007) and relatively high concentrations of ammonium (Schmidt et al. 2004; Beier et al. 2004) compared to amounts reported from other temperate and arctic heaths (Raubuch and Joergensen 2002; Bernal et al. 2005; Weintraub and Schimel 2005b). As expected, there was a pronounced seasonal variation, with low concentrations of both ammonium and amino acid in the peak growing season, while in the period from May to August, the plant fine root biomass doubled (Figure 2). The general increase in amino acid concentrations from early to late summer (Figure 1) may be explained by increasing and qualitatively different root exudation of amino acids (Lesuffleur et al. 2007). Also from May to August, the initial decrease followed by increase in the microbial N biomass is similar to the changes in both amino acids and ammonium. This may be explained by high plant acquisition of ammonium and amino acids during spring and early summer growth, followed by a period of lower plant demand from August. The decrease in microbial biomass in late summer allows for the observed increase in plant root production and plant nutrient acquisition.

283 As we added a mixture of N forms, the ^{15}N uptake by the plants and microbes is likely to
284 reflect preference of specific N forms. Still, the uneven dilution of the added isotope label by the
285 labile N forms already present in the soil leads to an uneven ^{15}N and ^{13}C enrichment of the soils
286 pools of ammonium, glycine, glutamic acid and phenylalanine. This is unavoidable in a field
287 experiment (Andresen and Michelsen 2005; Sorensen et al. 2007; Kielland et al. 2007).
288 However, by a comparison on the scale of ^{15}N recovery (i.e. the proportion ^{15}N found per unit
289 area out of the total amount added ^{15}N per square meter, Table 3) differences in amounts of
290 added ^{15}N can be ignored.
291 The major part of the added ^{15}N label was recovered in the top 0-5 cm the soil layer,
292 suggesting small losses by leaching during the first day of label distribution. Hence, the ^{15}N
293 recovery in the different pools after one day is an indication of the N uptake and shows the
294 short-term pattern of N uptake by microbes and plants. Overall, the plant:microbial ^{15}N recovery
295 ratio was 1:12, and the microbes were, consequently, superior to plants in the short term N-
296 uptake, in correspondence with our hypothesis. Compared to plants, the microorganisms hold a
297 smaller biomass, N-pool and C-pool, so the different uptake patterns illustrate different
298 acquisition strategies of the these organism groups, with no correspondence of mass or N-pool
299 dominance and acquisition (McKane et al. 2002; Sorensen et al. 2007; Harrison et al. 2008). As
300 both plants and microorganisms show capability for uptake of ammonium and intact amino
301 acids from the same pools, rapid fluxes, high uptake rates and alternating mineralization and
302 immobilization of nutrients in plants and microbes are important elements of nutrient cycling in
303 terrestrial ecosystems.
304 The soil microorganisms took up the added N-forms with no significant preference
305 (Table 3), and showed uptake of both ^{15}N and ^{13}C (Figure 5). The significant linear regression of

306 ^{15}N- and ^{13}C enrichments of the microbial biomass and the stoichiometry show, as hypothesized,
307 that the amino acids were absorbed as intact compounds by the soil microorganisms. This agrees
308 with similar findings in other ecosystem types (Näsholm and Persson 2001; Nordin et al. 2004;
309 Harrison et al. 2008).
310 Similarly, the linear relationship between ^{13}C and ^{15}N and the high ^{13}C :^{15}N ratio of the
311 grass and *Calluna* roots in the phenylalanine labelled plots strongly suggest uptake of intact
312 phenylalanine, similar to reported results from other ex-situ studies (Watson and Fowden 1975)
313 and field studies in other ecosystem types (Nordin et al. 2004; Hofmockel et al. 2007; Harrison
314 et al. 2008). In *Calluna*, the amount of carbon from phenylalanine incorporated into the roots
315 corresponded to 0.02‰ and in grasses to 0.04‰ of the root carbon pool on the field site. In
316 contrast, there was no significant^{15}N:^{13}C relationship in grass and *Calluna* roots from the plots
317 treated with glycine and glutamic acid, suggesting that the amino acids were not acquired as
318 intact compounds (Andresen and Michelsen 2005; Rains and Bledsoe 2007).
319 The ^{15}N concentration in *Calluna* tissue gradually decreased from fine roots, through
320 coarse roots and coarse branches to the lowest concentration in the green shoots, illustrating the
321 advancing translocation of the absorbed nitrogen through the plant (data not shown). Hence,
322 already one day after soil labelling, the absorbed N reached the leaves and could be incorporated
323 in proteins and enzymes essential for e.g. photosynthesis. However, the enrichment in the shoots
324 of ^{15}N from ammonium was higher than the enrichment of ^{15}N from phenylalanine, suggesting
325 that the translocation of N from the amino acids acquired in intact form was slower than the N
326 from the ammonium uptake. In grasses, the concentration of ^{15}N from ammonium in roots and
327 shoots was similar. However, the concentration of ^{15}N from phenylalanine was larger in roots
328 than in shoots, suggesting a similar pattern as in *Calluna* with slower translocation of

329 phenylalanine than of ammonium. A delayed uptake of organic nitrogen by ericaceous species
330 as compared with inorganic N has also been reported from subarctic ecosystems and pygmy
331 forest (Andresen *et al.* submitted; Rains and Bledsoe 2007).

332 The short-term preference for NH_4^+-N rather than N from the amino acid sources by the
333 plants was evident from the higher ^{15}N recovery from ammonium than from the amino acids
334 (Table 3). Similar preference of inorganic nitrogen has also been reported from subarctic
335 (Sorensen et al., 2007) and temperate ecosystems (Hofmockel et al. 2007; Harrison et al., 2008).

336 There was a significant (P=0.0001) overall effect of plant species on ^{15}N recovery. The
337 recovery of ^{15}N label in *Calluna* and grasses was similar in ammonium and phenylalanine plots,
338 while the grasses took up more glycine and glutamic acid than did *Calluna* (Table 3). The ^{15}N
339 recovery in mosses was much lower than in vascular plants, presumably because uptake by
340 mosses mostly is from atmospheric N deposition.

341 Addition of tannic acid to the soil solution had only minor effects on the investigated
342 processes, in contrast to findings by (Holub and Lajtha 2004). The higher DOC, and lower DON
343 concentrations and ^{15}N-TDN recovery in the soil extracts from the tannic acid additions may
344 have been caused by complex binding of the tannic acid with specific organic compounds,
345 changing their extractability (Halvorson and Gonzales 2008). In support of this, the tendency
346 towards lower ^{15}N recovery in the total soil at 0-5 cm depth with added tannins suggests that
347 some organic compounds, complexed with tannic acid, to a higher extent had percolated to soil
348 layers below 5 cm depth, similar to processes observed by (Holub and Lajtha 2004).

349 The effect of tannic acid on the recovery of ^{15}N was non-significant in soil
350 microorganisms, grasses and mosses. However, in *Calluna* tannic acid addition reduced the
351 recovery of some of the added N forms, shown by the significant tannic acid*N form interaction

(Table 3). For instance, the ^{15}N enrichments in green shoots and coarse branches of *Calluna* were both 62% higher in ^{15}N ammonium plots without than with addition of tannic acid (data not shown). Likewise, in plots with labelled glycine and phenylalanine, *Calluna* showed higher ^{15}N enrichment in the fine roots and in plots with phenylalanine also in coarse roots. The more pronounced response to tannic acid in *Calluna* than in graminoids may be due to the different ammonium and amino acid transporters in root and in mycorrhizal fungi (Fischer et al. 1998; Chalot et al. 2002; Svennerstam et al. 2007).

The absence of tannins in the leaves of the graminoids together with the ^{13}C and ^{15}N uptake from phenylalanine, suggest that the acquired phenylalanine is utilized for protein and not secondary compound synthesis in the graminoids. By contrast, the high concentration of condensed tannin in *Calluna* leaves and roots together with the ^{13}C and ^{15}N uptake from phenylalanine, suggests that phenylalanine may be utilized for both protein synthesis and for synthesis of secondary compounds in *Calluna* and, hence, different fate of added phenylalanine for these two heathland plant species, in correspondence with the protein competition model (Jones and Hartley 1999; Kraus et al. 2003).

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Figure legends

Fig 1: Seasonal variation in free amino acids in the heathland soil (μg N g^{-1} SOM), means shown with standard error. Arg: arginine, Lys: lysine, Gln: glutamine, Asn: aspargine, Ala: alanine, Gly: glycine, Val: valine, Ser: serine, Prol: proline, Ile: isoleucine, Leu: leucine, Met: methionine, His: histidine, Phe: phenylalanine, Glu: glutamic acid, Asp: aspartic acid, Cys: cysteine, Tyr: tyrosine, Trp: tryptophan. Below 1. axis significant effect (one way ANOVA) of sampling time; P < 0.001: ***; P <0.01: **; P<0.05: *; P<0.1: †; P>0.1: ns; nd not determined.

Fig 2: Seasonal variation in microbial nitrogen (MicN) and ammonium NH_4^+-N in heathland soil (μg N g^{-1} SOM), means shown with standard error. Means with different letters are significantly different (one way ANOVA followed by Tukeys test α=0.05).

Fig 3: Seasonal variation in fine root biomass (g m^{-2}) of *Calluna vulgaris* and grasses from 0-5 cm depth, means shown with standard error. Means with different letters are significantly different (one way ANOVA followed by Tukeys test α=0.05).

Fig 4: ^{15}N enrichment (μmol ^{15}N m^{-2}) versus ^{13}C enrichment (μmol ^{13}C m^{-2}) in plant fine roots from **a)** graminoids and **b)** *Calluna vulgaris*, sampled at 0-5 cm depth one day after labelling with $^{15}N^{13}C_9$-phenylalanine with and without tannic acid. Linear regression forced through zero, n = 12.

Fig 5: ^{15}N enrichment (μmol^{15}N m^{-2}) versus ^{13}C enrichment (μmol^{13}C m^{-2}) in microbial biomass sampled at 0-5 cm depth one day after labelling with **a)** ^{15}N-ammonium with and without tannic

acid, **b)** $^{15}N^{13}C_9$-phenylalanine with and without tannic acid, **c)** $^{15}N^{13}C_2$-glycine with and without tannic acid and **d)** $^{15}N^{13}C_5$-glutamic acid with and without tannic acid. Linear regression forced through zero, n = 12. Data from plots with tannic acid added are indicated with filled symbols.

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Figure

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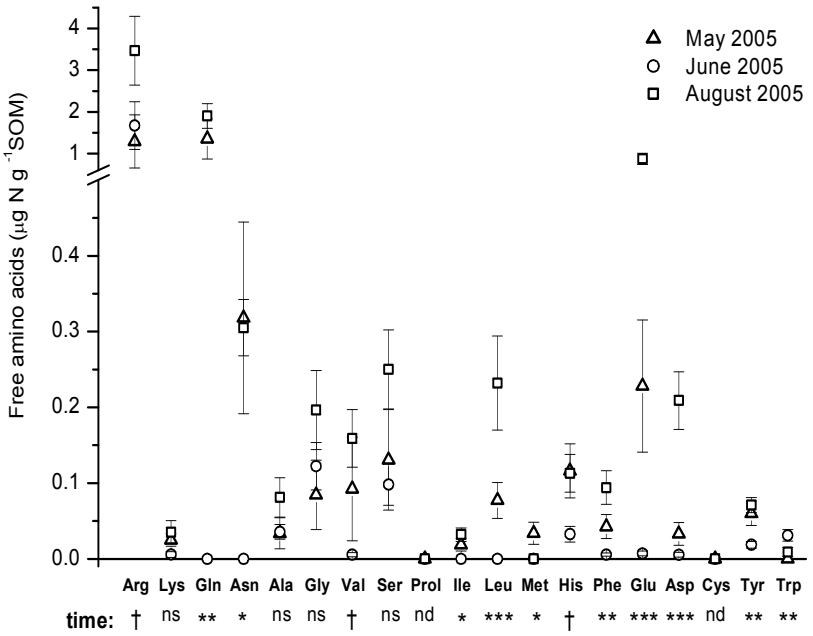

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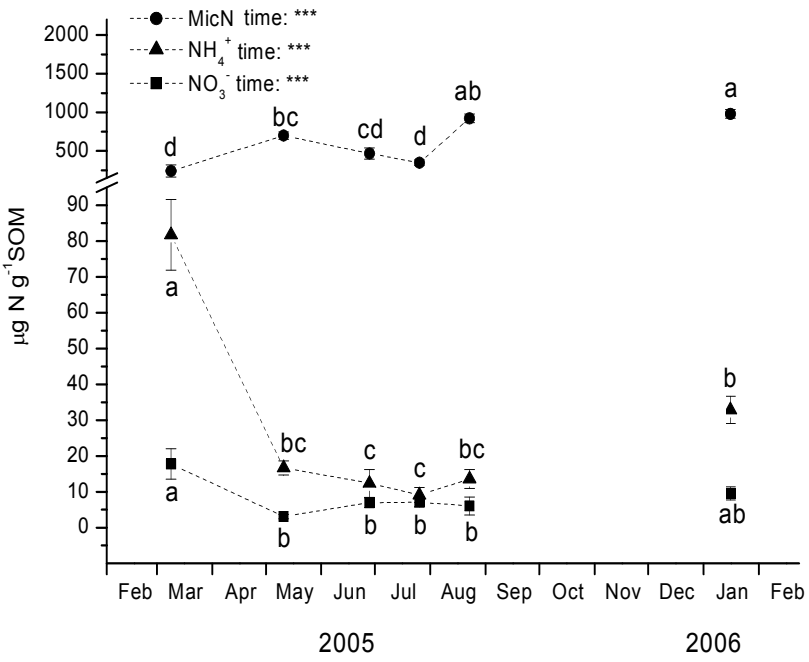
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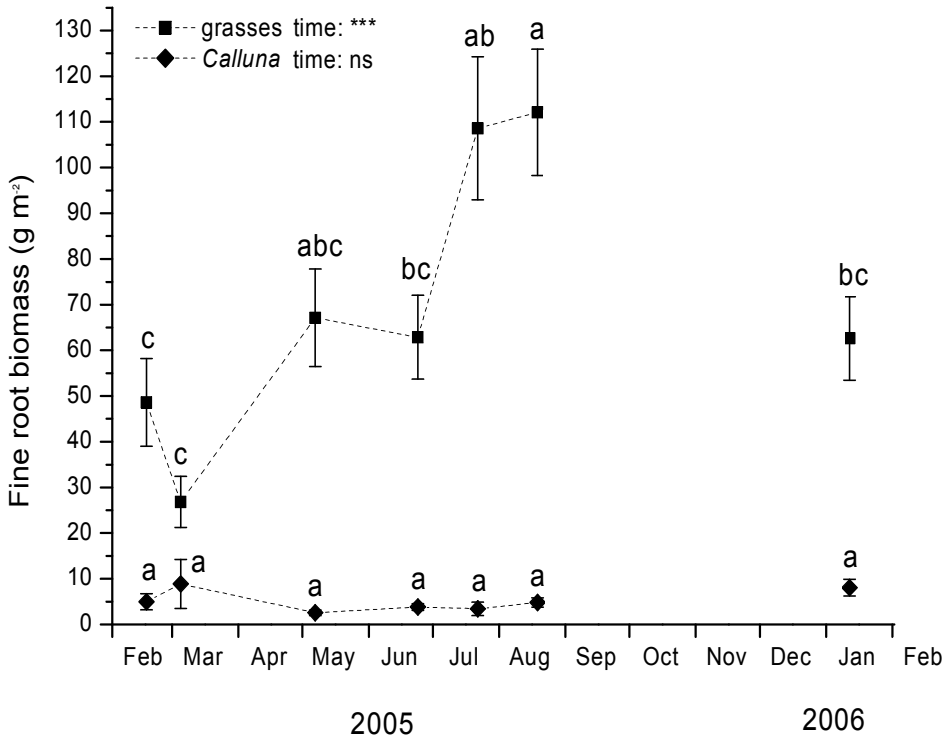

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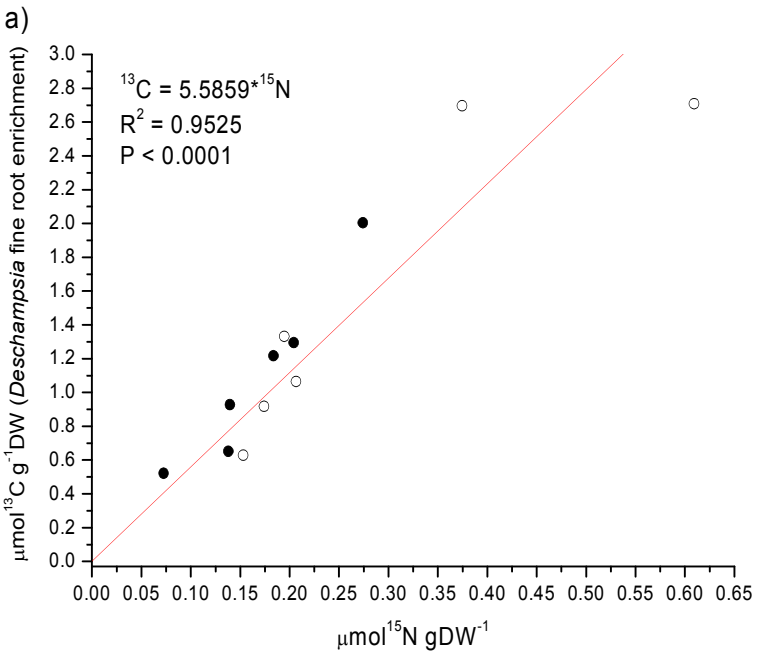

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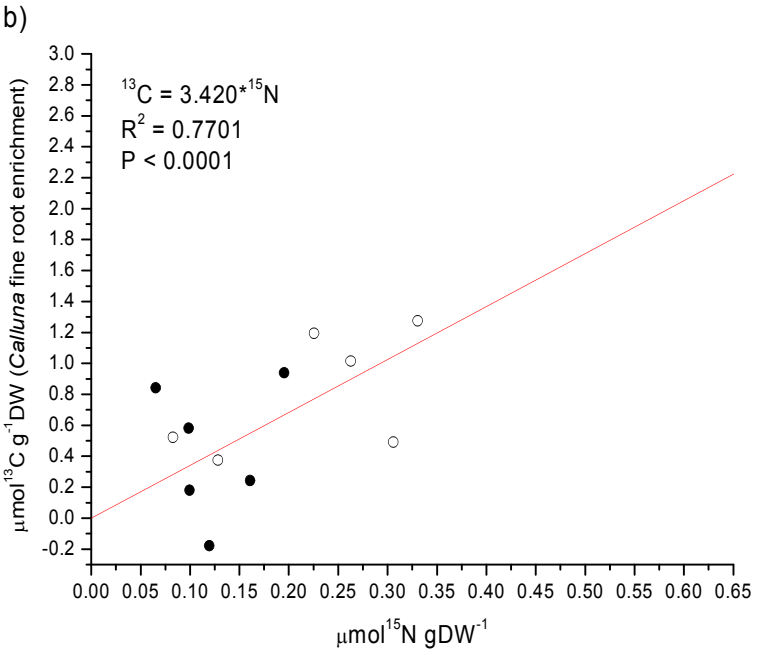

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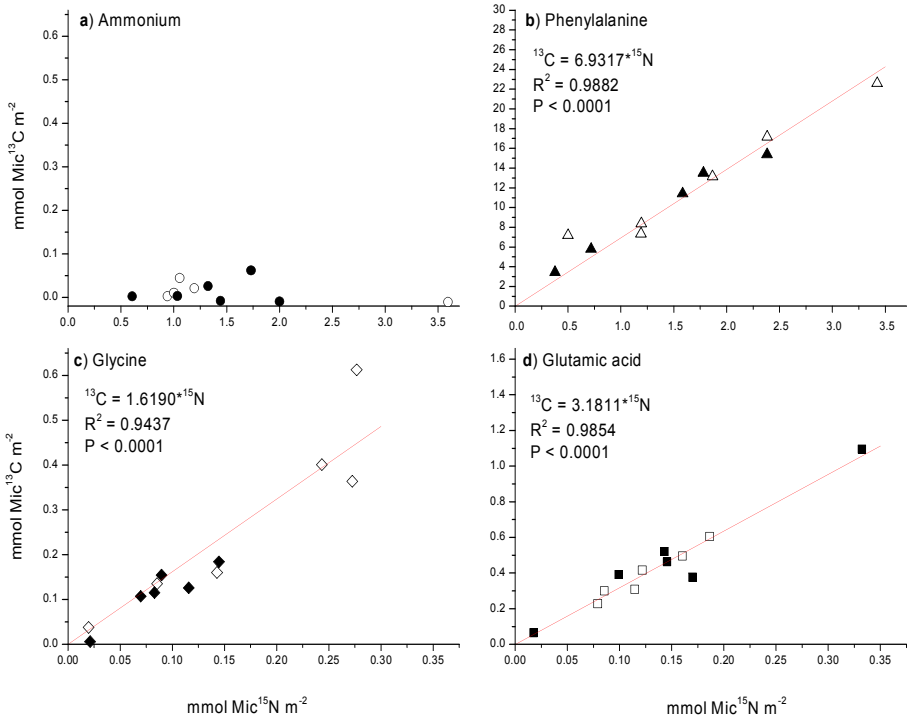

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Table 1: Soil properties (0-5 cm depth, n = 48) May 18, 2005 at the temperate heathland.

	mean $\mu g \cdot g^{-1}$SOM	se	mean $g \cdot m^{-2}$	se
NO_3-N	1.49	0.75	0.001	0.000
NH_4-N	8.12	1.37	0.008	0.001
DON	60.12	2.41	0.065	0.004
MicN	764.73	23.73	0.831	0.042
DOC	712.92	35.27	0.808	0.063
MicC	7858.14	231.71	8.508	0.407
Total amino acid-N	0.24	0.04	0.001	0.000

Table 2: Plant biomass (n = 48) May 18, 2005 at the temperate heathland. The belowground biomass is for 0-5 cm depth.

Aboveground	mean DW $g \cdot m^{-2}$	se	mean $gN \cdot m^{-2}$	se	mean $gC \cdot m^{-2}$	se
Calluna vulgaris green shoots	249.1	22.3	3.6	0.3	113.4	9.2
Calluna vulgaris coarse branches	198.5	21.7	1.6	0.2	88.7	10.8
Graminoid shoots	122.7	12.3	1.3	0.1	54.1	5.3
Mosses	60.7	11.8	1.2	0.2	31.2	6.4
Other	12.4	3.6	n.d.	n.d.	n.d.	n.d.
Total aboveground biomass	643.3	41.9	7.8	0.6	283.9	21.8
Litter all species	135.1	15.3	n.d.	n.d.	n.d.	n.d.
Below ground						
Calluna vulgaris coarse roots	234.3	37.5	1.5	0.3	90.5	16.8
Calluna vulgaris fine roots	34.3	3.9	0.3	0.0	16.7	2.0
Graminoid roots	336.6	34.2	3.4	0.4	145.7	16.5
Total belowground biomass	596.7	38.1	5.2	0.5	250.8	20.0

Table 3: ^{15}N recovery (% of added ^{15}N) in whole plants (*Calluna vulgaris*, grasses, mosses) and in soil microorganisms, in total dissolved N (TDN) and in total soil in 0-5 cm depth one day after ^{15}N labelling with four different N forms, with or without tannic acid (T) addition. Mean ± standard error (s.e.), n = 6). Results of two-way ANOVA are indicated, n.s. non-significant.

Treatment / ^{15}N recovery	***Calluna vulgaris***		**Grasses**		**Mosses**		**Soil microbial ^{15}N**		**Total dissolved ^{15}N**		**Total soil ^{15}N**	
	mean	se	mean	se	mean	se	mean	se	mean	se	mean	se
^{15}N ammonium	3.87	1.16	3.93	1.07	0.00	0.00	46.7	15.3	0.60	0.17	87.1	17.1
^{15}N phenylalanine	0.87	0.29	0.81	0.32	0.01	0.01	52.8	12.7	1.01	0.60	86.3	8.7
^{15}N glycine	0.66	0.24	1.21	0.44	0.02	0.01	52.0	13.2	0.75	0.10	76.6	24.2
^{15}N glutamic acid	0.57	0.21	1.26	0.40	0.01	0.00	37.4	5.1	1.34	0.35	88.6	20.3
T + ^{15}N ammonium	1.64	0.57	3.37	1.67	0.00	0.00	40.7	6.1	0.88	0.53	73.4	8.8
T + ^{15}N phenylalanine	2.20	0.44	2.45	0.98	0.00	0.00	41.0	10.9	0.12	0.02	73.7	20.5
T + ^{15}N glycine	0.51	0.15	2.39	0.88	0.00	0.00	26.2	5.1	0.24	0.04	44.8	10.3
T + ^{15}N glutamic acid	0.40	0.39	1.38	0.46	0.03	0.02	45.4	12.7	0.58	0.16	78.1	21.5
ANOVA												
Nform P-value	0.0014		0.042		n.s.		n.s.		n.s.		n.s.	
Taddition P value	n.s.		n.s.		n.s.		n.s.		0.0471		n.s.	
Nform***T**addition P-value	0.0478		n.s.		n.s.		n.s.		n.s.		n.s.	

Plant nutrient mobilization in temperate heathland responds to drought, elevated temperature and CO_2

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Abstract

- Temperate terrestrial ecosystems are currently exposed to changes in climate with increased atmospheric CO_2, increased temperature and periodical droughts with consequences for natural ecosystems and the potential feedbacks to the climate. We here present results from a novel field experiment, where the effects of these three climate change factors are investigated solely and in all combinations at a temperate heath dominated by *Calluna vulgaris* and *Deschampsia flexuosa*.
- Responses in soil inorganic and microbial nutrient concentration were investigated in the second year of treatment. Net mineralization and immobilization in the top soil and leaf litter decomposition was investigated through the winter season separately below *Calluna* and *Deschampsia* plants respectively with different responses for the two species.
- After one year of treatment, warming increased microbial C, N and P at 0-5 cm depth and decomposition of leaf litter below *Calluna* plants. The effects of warming were often counteracted when combined with CO_2 and drought.
- Net mineralization of N and P was significantly affected by the climate change treatments. In *Deschampsia* soil the net nitrification rate decreased significantly in response to drought, but an increase was observed in *Calluna* soil. By contrast, drought reduced leaf litter decomposition for both species.
- Soil incubations with plants present showed increased microbial immobilization of N relative to incubations without plants, suggesting a plant root exudation priming of the rhizosphere. Warmed plots with lower DOC concentrations had

lower mineralization rates, also suggesting a carbohydrate limitation of the microbes.

- Plant mobilization of N followed the observed responses in N mineralization due to plant acquisition of DIN. Furthermore, *Deschampsia* plants had larger nitrate acquisition than *Calluna* and *Calluna* showed preference of ammonium over nitrate.

Keywords: *Calluna vulgaris*, carbon, climate change, *Deschampsia flexuosa*, immobilization, microbial biomass, mineralization, nitrification, nitrogen, warming.

Introduction

Natural ecosystems respond to changes in air and soil temperature, atmospheric CO_2 concentration and drought, with consequences for biological processes and functioning. According to extrapolations and models developed by IPCC the air temperature may increase by 0.1 ˚C for each following decade, the CO_2 concentration of the atmosphere will increase with an amount depending on stabilization scenario. Furthermore precipitation will alter, with expected extended summer drought periods in Denmark (IPCC, 2007); (Danish Meteorological Institute, 2008). Investigations of the combined effects of increased temperature (T), CO_2 and drought (D) are necessary to reveal the actual responses (Mikkelsen *et al.*, 2008; Beier *et al.*, 2004a; Finizi *et al.*, 2006).

The significance of plants and soil microbial biomass as carbon sinks and processors of soil organic matter respectively (Rustad et al. 2001; Beier *et al.* 2004a; (Emmett *et al.*, 2004; Beier *et al.*, 2004a) Peñuelas et al., 2004; (Finizi *et al.*, 2006; Norby & Iversen, 2006)), relates strongly to the factors limiting the organisms, such as the

availability of nitrogen, and phosphorus, labile carbon or water. Changes in nutrient cycling in the ecosystem as direct or indirect response to climate alterations may in the long term bottom up control the ecosystem carbon sink response, eventually by progressive nitrogen limitation (Luo *et al.*, 2004; Norby & Iversen, 2006; Finizi *et al.*, 2006; Hungate *et al.*, 2006).

Soil microbial processes evidently respond to climate changes, with ecosystem type specific direction of the responses. Generally, net nitrification and mineralization rates and leaching of inorganic nitrogen, increased in response to warming and drought (Rustad *et al.*, 2001); (Jonasson *et al.*, 2004); (Rinnan *et al.*, 2006). Microbial biomass in dry *Calluna* heathlands decreased in response to drought (Jensen *et al.*, 2003), while microbial immobilization in tundra increased in response to warming (Schmidt *et al.*, 2002). Furthermore, litter decomposition generally increased in response to warming in subarctic ecosystems (Cornelissen *et al.*, 2007). Net N mineralization was significantly lower in grassland soil exposed to a gradient CO_2 treatment through three years, explained by a gradual decreasing substrate quality of the remaining soil organic matter (Gill *et al.*, 2002). However, no changes have been found for grass leaf litter decomposition in response to increased CO_2 (de Graaff *et al.*, 2006; Knops *et al.*, 2007). Hence, responses to elevated CO_2 may be in opposite directions of responses to warming and drought. Furthermore, the responses in field investigations are often small compared to the natural seasonal variation, when investigated in temperate heath ecosystems (Anderson & Hetherington, 1999); (Schmidt *et al.*, 2004; Emmett *et al.*, 2004); (Beier *et al.*, 2004b; Beier *et al.*, 2004a); (Sowerby *et al.*, 2005).

The combined effects of warming, increased atmospheric CO_2 and summer drought on the soil processes of a temperate heathland ecosystem have not previously been investigated (Mikkelsen *et al.*, 2008). In the present study soil N and P mineralization, microbial immobilization and decomposition was investigated in buried bags and litterbags in a temperate heath ecosystem in order to reveal climate change effects on nutrient cycling. Furthermore, plants were introduced in the buried bags to study the processes with and without the presence of plants.

It was hypothesized, that in the short term:

- Biological processes would be stimulated by increased temperature (T) leading to increased net rates of nitrification, mineralization and decomposition as well as increased microbial C, N and P.
- Decomposing microorganisms would be water limited by the drought treatment (D) leading to reduced mineralization, nitrification and decomposition in response to drought.
- Plant presence will induce microbial immobilization of N and acquire mineralized nitrogen. Furthermore, T and CO_2 would increase the plant biomass due to increased photosynthesis and increase plant uptake of N.
- Elevated CO_2 will not affect soil mineralization and litter decomposition on the short term (< 2 years).

Methods

The field site

The field site for the investigation covered an area of about two hectares at Brandbjerg (55º53'N 11º58'E) a hilly nutrient poor sandy deposit with a dry heath/grassland ecosystem dominated by *Deschampsia flexuosa* and *Calluna vulgaris* and with a low cover of other herbs and grass species, and an open moss cover beneath the canopy of vascular plants. The average precipitation per year was about 600 mm and the average temperature was 8° C (www.dmi.dk, 2005).

The climate change manipulations

The climate manipulations started October 2005 and consisted of increased temperature (T), extended summer drought (D), increased atmospheric CO_2 and all combinations of these treatments (TD, TCO_2, DCO_2 and $TDCO_2$), all with a replication of 6. The study plots consisted of 12 octagons each 7 m in diameter. Each block comprised 2 octagons, one with CO_2 and one without CO_2. Each octagon comprised 4 plots in a split plot design with the treatments drought or elevated temperature solely or in combination, and a non-warmed, non-drought plot (Mikkelsen *et al.*, 2008). The temperature was increased by passive nighttime warming by means of low automatic curtains that rolled over the vegetation during night. To avoid changes in precipitation, the curtains were automatically removed during rain events. The precipitation in the drought plots was altered also with automatic curtains that automatically unfolded during rain events in early summer. The atmospheric CO_2 was increased with pipe fumigation as in a regular FACE experiment, and with a feed back control system linked to wind speed and wind

direction. The temperature increase of the soil in 2 cm depth was around 1˚C, the increased CO_2 concentration in the air was 510 ppm. The drought period started in late June 2006 and continued for 5 weeks until early August when soil water reached c. 5 vol% water in the top 20 cm of the soil. For further information about the experimental design of the multifactor set up, see Mikkelsen et al 2008.

Soil incubation in buried bag

Soil chemistry and mmineralization was investigated below both *Calluna* and *Deschampsia* plants in all 48 plots. In November 2006, one year after treatments were initiated, two intact block of soil (20×20 cm) one from below *Calluna* plants and one from below *Deschampsia* plants was cut from each plot. One subsample was directly used for analysis of initial soil properties. Other subsamples were carefully cut down to sizes of 4×4.5 cm carefully fitting into the plastic pots used for the incubation. The incubated soil was from the top of the turf without removal of any litter or roots and down to 5 cm depth. It was carefully slipped into the incubation pot with no compression. A lid of parafilm closed the pot but had a small slit to allow for plants in those with plant and to allow for the same water vapour exchange conditions in those without plant (Eno, 1960; Schmidt *et al.*, 2002). Each incubated subsample was cut in two vertically. Soil sampled below the two dominant plant species, viz. *Calluna* and *Deschampsia*, was incubated separately. For each plant type one sample was incubated soil alone and two similar (for sake of poor plant survival) incubations were made with soil with small *Calluna vulgaris* respectively *Deschampsia flexuosa* plants. The plants had been pre-grown from seeds (*Deschampsia*) and cuttings (*Calluna*) for a period of 2 and 15 months respectively in soil from the site prior to the incubations. Three *Deschampsia* seedlings

154 (0.08 g FW each) were planted in each pot with *Deschampsia* plant incubations, and two
155 *Calluna* seedlings and one cutting (0.05 g FW each) were planted in each pot with
156 *Calluna* plant incubation.
157 The incubation pots were placed in holes in the study plots in level with the
158 surrounding soil. A 10 cm tall net was tightened around the pots to exclude mice. After
159 half a year of incubation in May 2006 after winter, the pots were sampled for analysis.
160 The initial soil samples and the sampled buried bag incubations after half a year were
161 kept cold until sorted. The small plants were carefully removed, and roots and litter was
162 sorted manually from the samples. Water content was measured after drying at 80˚C and
163 soil organic matter as loss on ignition after 550˚C for 6 hrs.

164 **Leaf litter incubation in litter bags**

165 Ambient leaf litter (standing dead biomass) from *Calluna* and *Deschampsia* was
166 collected at the area of the field site in February 2006. The *Deschampsia* leaf litter was
167 only current year leaf and straw litter, still attached to the plant. It was dry at collection
168 and kept in refrigerator. The *Calluna* leaf litter was collected by shaking the *Calluna*
169 shrubs, hence it was assumingly current year leaf litter. The litter consisted of 27% (by
170 dry weight) flowers, 36% leaves and small branches, 19% branches larger than 5 mm in
171 diam. and 18% mixed un-definable material.
172 The litter was cut down to lengths no longer than 3 cm. It was then incubated in
173 4×4 cm litterbags. Bags with *Deschampsia* litter had a mesh size of 1×1 mm and *Calluna*
174 litter had a mesh size of only 0.05×0.05 mm to ensure that no small leaves would drop
175 out. The *Deschampsia* litterbags each had 1.0 g FW litter and the *Calluna* litterbags each

had 2.0 g FW litter. The litterbags were placed at the soil surface below the plant species of origin, and fixed with a small plastic pin and covered with the litter at the spot.

The litter incubation started March 20th 2006 and bags were collected after 214 and 381 days (*Calluna*) and 214, 381 and 458 days (*Deschampsia*). The collected bags were frozen until sorted for grown-in mosses and plant roots. The litter was then freeze dried. The litter massloss was calculated as:

$$\text{mass loss \%} = 100\ \% * (DW_{initial}-DW_{sample})/DW_{initial}$$

Chemical analysis and calculations

The fresh soil was extracted with 0.1 M K_2SO_4 (1:5 soil:water) for analysis of nitrate, ammonium, dissolved organic carbon (DOC) and dissolved phosphorus (P). Total dissolved nitrogen (DON) was analyzed after digestion of the extract with potassium peroxide sulphate. A subset of samples were fumigated with chloroform and extracted with 0.1 M K_2SO_4 for subsequent measurement of microbial carbon, phosphorus and, after digestion, microbial nitrogen.

The sorted roots and the incubation plants were washed and dried at 80˚C for three days and weighed. Digestion of dead roots and plants was with 1 ml H_2O_2, 5 ml H_2SeO_3 and 94 ml H_2SO_4 for 1 hr at 400˚C (Jonasson *et al.*, 2004).

N and P in extracted and digested samples were measured on Hitachi U 2010 Spectrophotometer. C was measured on a Shimadzu TOC 5000A analyzer. The microbial C, N, and P fractions were calculated assuming extractability factors of 0.40, 0.45 and 0.40, respectively (Schmidt *et al.*, 2002; Joergensen & Mueller, 1996; Joergensen, 1996; Schmidt *et al.*, 2004), and were normalized by sample soil organic matter content (SOM).

198 Net mineralization rates and rate changes in microbial C, N and P and in dead root
199 N and plant N were calculated as the difference between the concentration of the
200 incubated soil and the initial values (Beier *et al.*, 2004b; Emmett *et al.*, 2004). Hence for
201 nitrate, ammonium, dissolved organic N and microbial N the net rate was calculated as:
202
203 (sample(μgN g^{-1} SOM) - initial(μgN g^{-1} SOM)) / days of incubation(187 days);
204
205 A positive rate for nitrate-N is referred to as nitrification, a positive rate for ammonium-N
206 is referred to as mineralization. A positive change of microbial N or P is termed microbial
207 immobilization.
208 Nitrification, mineralization, DON production, microbial immobilization, dead
209 root N change, dead root mass change and small plant rate change in N and in biomass
210 were also calculated per incubation unit (core) for possible comparisons.
211 Treatment responses (e.g. drought, temperature or CO_2) for all measured
212 parameters was calculated as:
213
214 (Mean values all plots with the treatment) / (Mean values all plots without the treatment)
215

216 ## Statistical analysis

217 One-way analyses of variance (ANOVA) were used to test differences between plant
218 specific soils in ambient plots (*Calluna* or *Deschampsia* soil). Correlations of N, C and P
219 mineralization rates were tested with Kendall and Pearson product moment correlation.

220 Linear mixed models were applied to analyse the responses in SAS 8.0. Random effects
221 terms were block, treatment plot and octagons, respecting the nested structure of the
222 design. Main effects terms were the treatment factors: CO2, temperature (T), and drought
223 (D). All interaction terms between the factors CO2, D and T were included. The models
224 were gradually simplified, starting with the third order interaction, taking out non-
225 significant terms until only significant ($P<0.05$) or close to significant ($0.05<P<0.10$)
226 terms remained. Homogeneity of variances was investigated with residual plots and
227 appropriate transformations done if necessary (SAS Institute Inc., 2003).

228 *Results*

229 The soil properties of the ambient plots (Table 1) were not significantly different below
230 the two species, however, after one full year of climate treatments significant responses
231 to the main factors CO_2, T and D and interactions were observed (Table 2), and the
232 responses differed for the two plant soil types. Consequently, the chemical and microbial
233 properties of the incubations with *Calluna* soil and *Deschampsia* soil were initially
234 different and incubations of the two soils responded differently to the climate change
235 factors. No significant correlations were found between net N mineralization and P
236 mineralizations. The microbial C to N ratio represent a microbial community composed
237 by a mixture of fungi and bacteria (Jensen *et al.*, 2003; Sowerby *et al.*, 2005), and did not
238 change significantly in response to the climate treatments.
239 Plant survival in the buried bags was 98%. Overall the *Deschampsia* plants in the
240 incubations doubled their biomass while *Calluna* plants did not gain much mass. When
241 plants were present in the incubations the DIN production (NO_3 plus NH_4 production)
242 was significantly reduced both in *Calluna* ($P=0.0065$) and *Deschampsia* soil ($P<0.0001$)

(Figure 2). The overall effect of plant presence was an increase in microbial N immobilization rate, by tendency for *Deschampsia* soil (P=0.0855) and,not significantly, for *Calluna* soil.

Responses to drought

The mobilization of soil nitrogen showed strong responses to drought, with opposite directions for the two soil types and with significant effects of plant presence.

Drought reduced *Deschampsia* leaf decomposition after half a year (D: P=0.0333, T*D: P=0.0116) and *Calluna* leaf decomposition after one year (P = 0.0331, Table 2). Also, the net nitrification rate was reduced by drought in *Deschampsia* soil (no plants P = 0.0109) (Figure 1), while in contrast, the net nitrification rate (P=0.0925) and production of dissolved organic N (DON) (D: P=0.0766; D*CO2: P=0.0340) in *Calluna* soil tended to be stimulated (no plants) (Figure 1).

Drought tended to reduce *Deschampsia* root biomass (P=0.0634) and total plant biomass (P=0.0774, Table 2) and reduced total plant N (D: P = 0.0106, T*CO2: P= 0.0999) (Figure 2) while in contrast, drought tended to increase *Calluna* shoot biomass (P=0.0794, Table 2), and total plant N (T: P=0.0001, D: P=0.0004, T*D: P=0.0234, T*CO2: P=0.0681, T*D*CO2: P=0.0652) (Figure 2).

Responses to warming

The soil processes responded to elevated temperature (T), differently below the two species. Warming tended to stimulate *Calluna* leaf decomposition after one year (P=0.0988) (Table 2). Furthermore, warming reduced dissolved organic C (DOC)

(P=0.0349, Table 2) and the net mineralization rate (P=0.0190) in *Deschampsia* soil with plants (Figure 2).

The microbes in *Calluna* soil had significantly higher N content in warmed plots (N: T: P=0.0396, T*D*CO2: P=0.0134), and tended to have higher biomass (C) (T: P=0.0613, T*D*CO2: P=0.0617) and P content (T: P=0.0750), but this was for MicN and MicC counteracted when both D and CO_2 were also imposed, in the triple factor interaction (Table 2). Warming reduced immobilization of N by microbes in *Calluna* soil after the half year incubation both without (T: P=0.0374, D*CO2: P=0.0943), and with plants (T: P=0.0407, Figure 1), while microbial immobilization of P in *Calluna* soil with plants was stimulated (T: P=0.0114, T*D: P=0.0091, T*D*CO2: P=0.0288, data not shown).

The *Calluna* root biomass tended to increase in response to T (P=0.0675, Table 2), and the N in *Calluna* plants increased in response to T (Figure 2).

Responses to increased CO_2

Direct main effect responses to increased CO_2 were limited, but CO_2 in combination with D or T often counteracted other responses.

CO_2 tended to stimulate *Calluna* leaf decomposition after half a year (P=0.0744, Table 2), while net phosphorus mineralization in *Deschampsia* soil without plants was reduced (data not shown). *Deschampsia* shoot biomass tended to increase in response to CO_2 (P=0.0716, Table 2).

In addition to the main effect of CO_2 microbial biomass C change responded to the treatments with P=0.0401 for T*D interaction in *Deschampsia* soil with no plants (data not shown). Furthermore, the net change in DOC responded to the interaction

T*D*CO2 in the following three soil types: *Calluna* soil with plant: P=0.0120, *Deschampsia* soil with no plants: P=0.0432 and *Deschampsia* soil with plant: P=0.0188 (data not shown).

Discussion

Drought works as suppressor of nitrogen cycling in *Deschampsia* soil

The soil below *Calluna* and below *Deschampsia* had different patterns of nutrient cycling, as expected from other studies investigating mineralization in soil below different plant species (van Vuuren *et al.*, 1992; van der Krift & Berendse, 2001; Gill *et al.*, 2006). In other investigations in temperate heathlands, N mineralization in soil below grasses and decomposition of grass litter was faster than for *Calluna* (van Vuuren *et al.*, 1992; van Vuuren *et al.*, 1993). Hence, a faster N cycling and a potentially stronger response to climate changes in soil below *Deschampsia* compared to soil below *Calluna*, may potentially control changes of the vegetation cover (van Vuuren *et al.*, 1992; Emmett *et al.*, 2004; Schmidt *et al.*, 2004; Weintraub & Schimel, 2005).

In *Deschampsia* soil, the decrease in net nitrification and litter decomposition in response to drought was reflected also in a decreased plant N uptake. These responses to drought were in accordance with our hypothesis, hence, drought works as suppressor of nitrogen cycling in the *Deschampsia* soil.

Also in *Calluna* soil drought reduced leaf litter decomposition. However, the trends towards a drought induced increase in net nitrification rate, change of DON

309 production and dead root decomposition, together with stimulated plant N mobilization
310 suggest an opposite response of the *Calluna* soil-plant system to drought. Moisture
311 limitation of *Calluna* leaf and soil organic matter decomposition has previously been
312 found (Emmett *et al.*, 2004; van Meeteren *et al.*, 2007), also with a natural climatic
313 gradient (of several field sites in Europe) of moisture primarily explaining the variability
314 of the net N mineralization and nitrification rates.

315 The soil incubations started immediately after the imposed summer drought,
316 meaning that the observed drought effects are related to the differences in the pre-
317 incubation history of the soil.

318 **Effects of elevated temperature on soil processes**

319 Pre-incubation differences were observed in the initial microbial biomass C, N and P pool
320 increases in response to T, with these initial differences in the incubations, possibly also
321 involving microbial community differences (Rinnan *et al.*, 2006), the microbial N
322 immobilization decreased and the leaf decomposition increased in response to T. In other
323 investigations at temperate heaths soil respiration and litter decomposition have been
324 shown strongly controlled by soil temperature (Emmett *et al.*, 2004). The findings in the
325 current experiment of warming causing a larger microbial biomass, higher leaf litter
326 decomposition and higher microbial release of N in *Calluna* soil, are in agreement with
327 other findings.

328 In our experiment, the initially smaller amount of DOC (total dissolved organic
329 carbon) in warmed plots occurred together with larger microbial biomass. This indicates,
330 that although the pool size of DOC is lower, the production probably is higher. Increased
331 microbial biomass has been related to higher microbial access to labile carbon (Schmidt

et al., 1997; 2000). In the warmed plots the 'missing' DOC could be due to a high demand and thus the measured DOC concentration showed no relation to the microbial N immobilization. Mineralization in the successive incubations decreased. Hence, we suggest that the soil mineralization processes require an ongoing carbohydrate supply for instance by plant root exudation, which was not available in the buried bags.

The decrease in mineralization in response to warming, has also been found in other mineralization studies of temperate heathland (Emmett *et al.*, 2004). This has most often been related to increased microbial immobilization in the bags (Schmidt *et al.*, 1999; Schmidt *et al.*, 2002) in contrast to the decrease in microbial N in this study. This, and the increase in phosphorus immobilization in response to warming did not support our hypothesis of increased mineralizations with elevated temperature. This may be due to the limited size of the incubated soil pools.

Plant uptake and mobilization of nitrogen

The observed increase in production rates of inorganic nitrogen (DIN) when plants are included in 'buried bag' studies (Figure 2) is in agreement with previous findings in a subarctic ecosystem (Jonasson *et al.*, 2004; Rinnan *et al.*, 2007). Both dominant plant species have nitrate reductase activity, with larger activity in *Deschampsia* compared to *Calluna* (Lee & Stewart, 1978; Högbom *et al.*, 2002; Troelstra *et al.*, 1995). Hence, *Deschampsia* plants have a greater potential for nitrate acquisition than *Calluna*. Furthermore, both species acquire ammonium, as previously found at a similar heath, with a larger ammonium acquisition by *Calluna* than *Deschampsia* during winter (Andresen & Michelsen, 2005). Such species specific plant acquisitions of nitrate and ammonium is supported by this study, with smaller nitrification and mineralization rates

355 in soil with plants, evidently due to plant acquisition. Consequently, *Deschampsia* plants
356 had a preference for nitrate, while *Calluna* showed preference of ammonium over nitrate.
357 The plant biomass and growth controlled the N uptake proportionally, and since
358 the soil-incubation study was carried out during winter, absence of growth and loss of
359 plant N by the *Calluna* plants was seen.
360 The larger microbial N immobilization in soil with plants compared to soil with
361 no plants is counter-intuitive (Rinnan *et al.*, 2007; Jonasson *et al.*, 2004), since plant
362 presence could be expected to lower the N availability for soil microbes. However, the
363 observations may be a response to increased plant carbohydrate root exudation, priming
364 the soil with labile substrate for the immobilizing microorganisms (Vestergård *et al.*,
365 2008). In this short term investigation, the plant control on DIN concentrations and
366 microbial N immobilization, and the species specific plant N responses to drought and
367 warming is a first indication of progressive nutrient mobilization by plants at this FACE
368 field site. Long term investigations may eventually show progressive nutrient limitation
369 of the natural vegetation (Luo *et al.*, 2004). Such a control pattern has not yet been found
370 in other soil mineralization studies (Finizi *et al.*, 2006; Norby & Iversen, 2006), where
371 the elevated CO_2 increased plant uptake of mobilized nitrogen. Direct effects of CO_2
372 treatment on the net mineralization in our study may appear in the longer term, as
373 suggested by the effects on *Calluna* leaf decomposition.
374

375 **Phosphorus may become a limiting factor**

376 The net mineralization rates of C, N and P were not significantly related, in contrast to
377 findings by Franzlubbers (Franzluebbers, 1999). The significant lower net P

mineralization rate in response to CO_2 and the altered P immobilization in this experiment, could reside from a CO_2 inhibition of the P mineralizing soil microorganisms, perhaps due to increased CO_3^- in the soil water solution in CO_2 fumigated plots. However, no CO_2 response in phosphatase in grasslands (Niklaus *et al.*, 2007; Menge & Field, 2007) has been found. This possible CO_2 inhibition of P availability, together with the previously observed increase in microbial immobilization of P in response to T in the *Calluna* soil incubated with plants, may eventually cause a P limitation of the heathland vegetation, as also found by van Meeteren et al. 2007. Phosphorus, hence, becomes a controlling factor of plant biomass carbon sequestration (a progressive phosphorus limitation), as has been found after nitrate addition in a similar T, precipitation and CO_2 manipulation experiment and in other global change experiments (Menge & Field, 2007).

Conclusions: climate change responses at the temperate heath

With different responses for *Calluna* and *Deschampsia* soil to elevated temperature, increased CO_2 and summer drought treatments had significant effects on soil C, N and P mineralization, microbial C, N and P immobilization, litter decomposition and plant growth.

Deschampsia soil responded to drought by a decrease in net nitrification and litter decomposition as well as reduced plant N uptake, meaning that the drought was a suppressor of nitrogen cycling. In *Calluna* soil these responses tended to be opposite.

Warming caused larger microbial biomass (C, N and P) and a larger litter decomposition and microbial release of N in *Calluna* soil. Furthermore, warming reduced

mineralization in *Deschampsia* soil and an increase in immobilization of P in *Calluna* soil.

Responses of soil mineralization to elevated CO_2 after only 1½ years were limited to a decrease in P mineralization. Additionally, *Deschampsia* responded with a larger shoot biomass.

Plants in the incubations mobilized and acquired inorganic N (NO_3^- and NH_4^+), with *Calluna* showing a preference for ammonium over nitrate and *Deschampsia* having larger nitrate acquisition than *Calluna*. Furthermore, plant presence increased the microbial immobilization, perhaps through priming of the rhizosphere soil.

In the short term, the investigated ecosystem processes were more responsive to drought than to increased temperature and CO_2. However, the combined effects of elevated temperature, CO_2 and drought often counteracted the main effects. Thus, the study emphasizes the need to investigate interactions between climate change factors as these may be unpredictable based only on single factor studies.

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Figure 1:

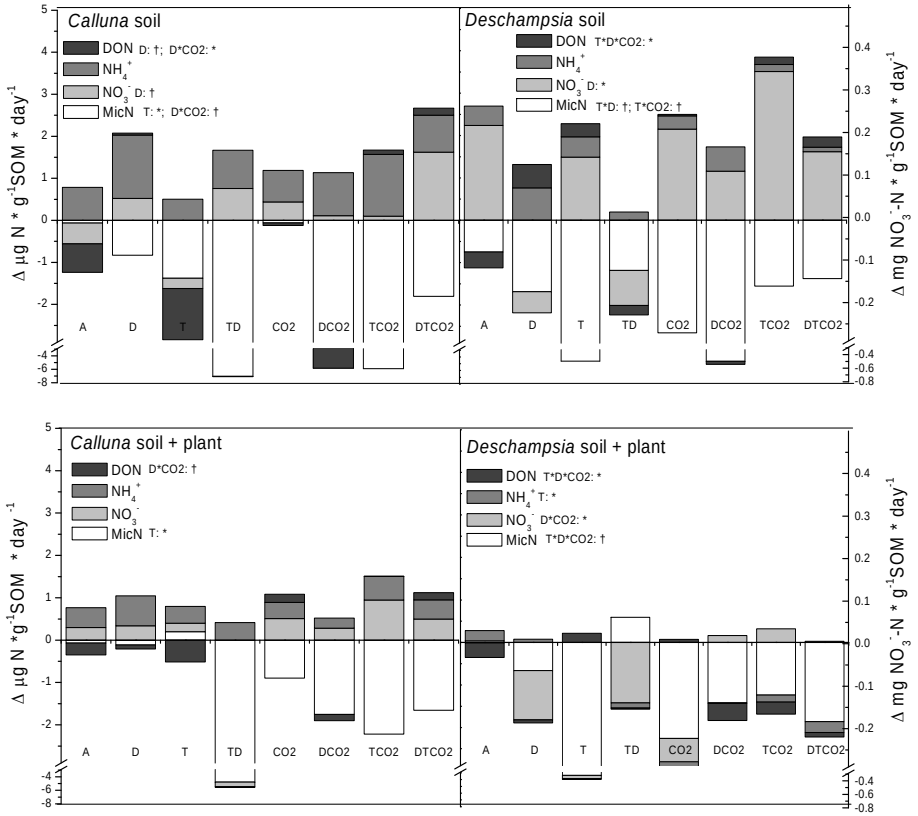

Figure 1: Changes in soil nitrogen pools: nitrification rate (ΔNO_3^--N, right 2nd axis), mineralization rate (ΔNH_4^+-N, left 2nd axis) and dissolved organic N production rate (ΔDON, left 2nd axis) and microbial N immobilization rate (ΔMicN, left 2nd axis) in units per g soil organic matter (SOM) per day, after incubation for a half year. Four variations of incubations: *Calluna* soil and *Deschampsia* soil, with no plant or with plant. Statistical significant effects from proc mixed model analysis of variances for the main effects: D, T and CO2 and the interactions D*T, D*CO2, T*CO2 and D*T*CO2 is indicated as follows: *** indicates $P < 0.001$; ** indicates $P < 0.01$; *: $P < 0.05$; †: $P < 0.1$.

Figure 2:

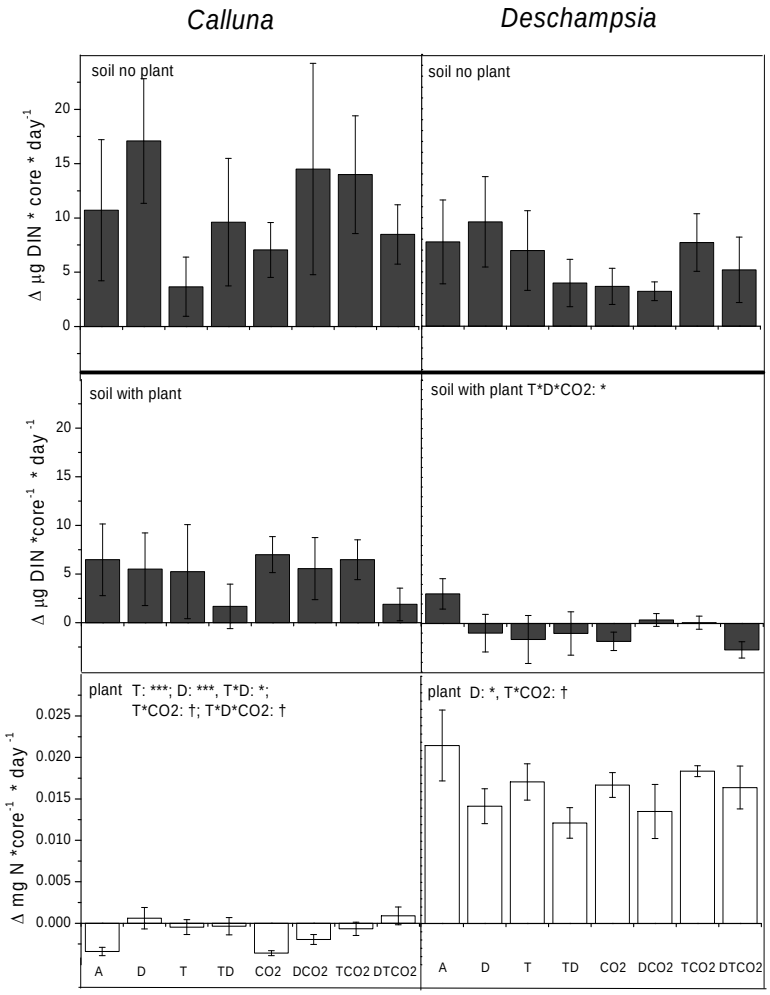

Figure 2: Changes in soil inorganic N (ΔDIN = ΔNO_3^--N plus ΔNH_4^+-N) (dark bars) and in plant N (open bars) per incubation core per day, through a half year. Four variations of incubations: *Calluna* soil and *Deschampsia* soil, with no plant or with plant. Statistical significant effects from proc mixed model analysis of variances for the main effects: D, T and CO2 and the interactions D*T, D*CO2, T*CO2 and D*T*CO2 is indicated as follows: *** indicates $P < 0.001$; ** indicates $P < 0.01$; *: $P < 0.05$; †: $P < 0.1$.

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584 **Table 1:** Soil properties below *Calluna* or below *Deschampsia* vegetation, plant biomass
585 of the small incubation *Calluna* and *Deschampsia* plants grown for half a year in the
586 treatments; and incubated *Calluna* and *Deschampsia* plant leaf litter mass loss. Mean
587 values and standard error (s.e.) for plots with no climate treatments.

588

Ambient plots						
			Calluna		***Deschampsia***	
			mean	**s.e.**	**mean**	**s.e.**
Soil properties	**SOM**	**%**	12.4	1.1	15.5	4.0
	NO_3-N	**µg*g^{-1} SOM**	29.9	24.9	5.6	2.1
	NH_4-N	**µg*g^{-1} SOM**	110.9	47.4	58.6	14.3
	DON	**µg*g^{-1} SOM**	180.1	122.1	115.5	79.0
	Microbial N	**µg*g^{-1} SOM**	1573.9	260.6	1320.8	92.1
	DOC	**µg*g^{-1} SOM**	724.5	87.6	904.8	109.5
	Microbial C	**µg*g^{-1} SOM**	9802.2	1384.6	7603.3	1776.1
	Dissolved P	**µg*g^{-1} SOM**	12.7	2.8	10.7	1.7
	Microbial P	**µg*g^{-1} SOM**	323.3	84.3	346.1	72.4
	Microbial C:N		6.2		4.8	
	Microbial N:P		4.9		3.8	
Plant biomass	**shoot**	**g**	0.046	0.005	0.105	0.025
	root	**g**	0.012	0.001	0.103	0.030
	root : shoot		0.260	0.018	0.922	0.212
	total plant	**g**	0.058	0.006	0.208	0.051
Leaf litter mass loss	**half a year**	**% loss**	25.79	1.31	33.39	1.09
	one year	**% loss**	34.28	1.42	45.14	4.28
	one year plus	**% loss**	.	.	46.05	4.67

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591

592 **Table 2:** The response effect for soil properties below *Calluna* or below *Deschampsia* in
593 plots after one year of climate treatments, representing initial incubation soil; response
594 effects for plant biomass of the plants incubated for half a year in the treatments; and for
595 litterbag incubated plant litter mass loss after ½, 1 and 1¼ years. The response effects are
596 for the main treatments drought (D), temperature (T) and CO_2 (CO2). Response is
597 calculated as: (means of plots with the treatment) / (means of the plots without the
598 treatment). Statistical significant effects from proc mixed model analysis of variances for
599 the main effects: D, T and CO2 are indicated as follows: *** indicates P < 0.001; **
600 indicates P < 0.01; *: P < 0.05; †: P < 0.1. Interactions (P < 0.05): D*T, D*CO2, T*CO2
601 and D*T*CO2 are indicated when significant.

602

603

Response		D	D	T	T	CO2	CO2	Significant interactions	
		Calluna	*Deschampsia*	*Calluna*	*Deschampsia*	*Calluna*	*Deschampsia*	*Calluna*	*Deschampsia*
Soil properties	**SOM**	0.83	0.89	1.01	0.78	0.90	1.05	.	.
	NO_3-N	0.69	1.57	0.93	1.46	0.44	0.54	.	.
	NH_4-N	0.58	0.99	0.64	1.38	0.54	1.05	.	.
	DON	0.60	1.20	0.87	0.80	1.84	1.33	.	.
	Microbial N	1.03	0.98	**1.23 ***	1.04	0.95	1.06	T*D*CO2	.
	DOC	1.00	1.04	0.95	**0.84 ***	0.81	1.04	T*D*CO2	.
	Microbial C	0.91	1.08	***1.15 †***	1.00	1.14	1.29	.	.
	Dissolved P	0.98	1.00	1.04	1.07	1.02	1.24	.	.
	Microbial P	0.97	0.99	***1.29 †***	0.97	1.10	1.23	.	.
Plant biomass	**Shoot**	***1.15 †***	0.8	1.1	1.0	1.0	***1.31 †***	.	.
	Root	0.93	***0.70 †***	***1.22 †***	1.04	1.17	1.09	.	.
	Root : Shoot	**0.85 ***	0.85	1.08	1.18	1.21	0.82	.	.
	Total plant	1.10	***0.79 †***	1.14	1.01	1.01	1.21	.	.
Leaf litter mass loss	**Half a year**	0.95	**0.89 ***	1.03	1.09	***1.04 †***	0.90	.	T*D
	One year	**0.93 ***	0.97	***1.06 †***	1.06	0.98	1.04	.	.
	One year plus	.	0.92	.	1.10	.	1.03	.	.

604

Glycine acquisition in temperate heath vegetation and soil microorganisms is influenced by elevated temperature, CO_2 and drought

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Abstract

- Temperate terrestrial ecosystems are currently exposed to climatic and air quality changes with increased atmospheric CO_2, increased temperature and periodical droughts. The responses of natural ecosystems and the potential feedbacks to the climate are intensely debated. We here present results from a unique field experiment, where the effects of these three climate change factors are investigated solely and in all combinations at a temperate heath dominated by *Calluna vulgaris* and *Deschampsia flexuosa*.
- In heath soil, free amino acids can serve as substrates for soil microorganisms and are acquired as nutrients directly by plants. Furthermore, amino acids are plant root exudates. In a future climate, plant productivity and plant root exudation may increase due to increased photosynthesis. In this experiment we investigated the distribution and uptake of $^{15}N^{13}C_2$-labeled glycine.
- Uptake of ^{15}N was 18 timers larger in the microbial biomass than in the plants. Hence, the soil microorganisms were superior to plants in the short term competition for the added nitrogen pulse. Soil microorganisms acquired glycine largely as intact compounds as shown by a ^{13}C to^{15}N ratio of 1.7. Plants showed no significant acquisition of intact glycine compounds.
- The *Deschampsia* root nitrogen acquisition responded significantly to the climate change treatments. Warming and CO_2 caused larger ^{15}N acquisition. However, this was counter-acted when the treatments were combined and additionally combined with drought. Furthermore, *Deschampsia* showed higher green leaf biomass and larger root N concentration in warmed plots with CO_2 added. This was reflected by lower nitrate concentration. We interpret this as altered senescence phenomena.

Introduction

Natural ecosystems respond to changes in air and soil temperature, atmospheric CO_2 concentration and drought, with consequences for biological processes and functioning of individuals and communities. According to extrapolations and models developed by IPCC the air temperature may increase by 0.1 ˚C for each following decade, and the CO_2 concentration of the atmosphere will increase with an amount depending on stabilization scenario. Furthermore the precipitation pattern will alter, with expected extended summer drought periods in Denmark (IPCC, 2007); (Danish Meteorological Institute, 2008). Investigations of the combined effects of increased temperature (T), CO_2 and drought (D) are necessary to reveal the actual responses (Mikkelsen *et al.*, 2008a; Beier *et al.*, 2004; Finizi *et al.*, 2006). There are few experiments in which the combined effects of CO_2 and warming have been studied, and none which combine these factors with drought. The current study presents data on plant N uptake and biogeochemical responses to the factors warming, elevated CO_2 and drought in a temperate heathland.

Soil microorganisms and plants acquire nitrogen from both inorganic (NO_3^- and NH_4^+) and organic sources (amino acids), and acquire intact amino acids (Nordin & Näsholm, 1997; Näsholm *et al.*, 1998; Persson & Näsholm, 2001; Hofmockel *et al.*, 2007). The free amino acids in the soil pore water origin partly from rhizo deposition (Lesuffleur *et al.*, 2007; Ström & Christensen, 2007) and partly as leachates from decomposing organic matter. Hence, amino acids in the soil function both as nitrogen sources and as labile carbohydrate substrates for soil microorganisms (Illeris & Jonasson, 1999; Ström & Christensen, 2007; Vestergård *et al.*, 2008).

Responses in root nutrient uptake to elevated CO_2 is highly variable, reflecting e.g. differential responses in plant growth and nutrient status, while plant processes such as water-use efficiency, photosynthetic rate (Ehleringer, 2005), tissue N-concentration and labile carbohydrates show consistent responses to elevated CO_2 (Bassirirad, 2000).

Various parameters reflecting root uptake kinetics are enhanced by warming, and the acquisition may increase by changed root transport properties for NH_4^+ (Clarkson &

Warner, 1979) though this exact mechanism is not clearly understood, and by changed fluidity of the phospholipids in root plasmalemma (Pike & Berry, 1980). Furthermore, NO_3^- uptake capacity is highly modulated by the N status of the roots or the whole plant (Bassirirad, 2000). Root biomasses, depth distribution and root morphology respond differentially to warming (Björk *et al.*, 2007). Consequently, the acquired N pool of the plant roots in response to warming is a combined effect of root biomass, nutrient status and root growth responses combined with physiological parameters affecting the acquisition.

Carbohydrate exudation by plant roots may respond to climate change in the same direction as photosynthesis and plant production (Rinnan *et al.*, 2005; Albert *et al.*, 2005; Ehleringer, 2005). Hence, elevated temperature and CO_2 may increase soil concentrations of e.g. glycine. In this experiment we investigated the acquisition and partitioning of glycine between plants and soil microorganisms. Glycine was labelled with the stable isotopes ^{15}N and $^{13}C_2$ and injected into the soil. The uptake of ^{15}N and ^{13}C was traced in samples of plant material from *Calluna vulgaris*, *Deschampsia flexuosa* (all with C_3 photosynthesis) and mosses and in soil microorganisms. Our aim with the investigation was to follow the potential organic nitrogen (in form of the amino acid glycine) acquisition by plants and soil microorganisms under climate change (Hofmockel *et al.*, 2007; Sorensen *et al.*, 2008b). Effects of one year of climate change treatments on soil mineral and organic N, microbial biomass C and N, and plant N acquisition was furthermore investigated.

In response to the climate change factors we expected:

- soil microorganisms would acquire the largest amount of the added glycine, with treatment responses in microbial ^{13}C and ^{15}N acquisition following the responses in microbial biomass
- warming to increase plant biomass and increase root ^{15}N uptake.
- elevated CO_2 to increase plant biomass and dilute tissue N concentration.
- nitrate concentration in sub-soils would respond to the climate change factors in opposite direction than plant biomass responses caused by plant nitrate acquisition.
- following this: increases in plant nitrogen demand, caused by the increased plant biomasses, would cause increased ^{15}N-glycine uptake.

Methods

The field site

The experiment took place at the site of the CLIMAITE experiment (Mikkelsen *et al.*, 2008b) at Brandbjerg (55º53'N 11º58'E) c. 50 km NW of Copenhagen, Denmark. The site was a managed, dry, temperate heath on a hilly nutrient-poor sandy deposit, with an organic layer of c. 5 cm depth and a pH of about 5. The vegetation was dominated by *Calluna vulgaris*, *Deschampsia flexuosa* and *Festuca ovina* accompanied by heathland mosses and herbs. The average precipitation per year was about 600 mm and the average temperature was 8° C (www.dmi.dk, 2005).

The climate change manipulations

The climate manipulations started October 2005 (Mikkelsen *et al.*, 2008b) and consisted of eight treatments: plots with increased temperature (T), altered summer drought (D), increased CO_2 concentration in the air and all combinations of these treatments (TD, TCO_2, DCO_2 and $TDCO_2$), plus control plots (A), all with a replication of 6. The field site covered an area of about two hectares and the experimental plots were distributed in 12 seven meter diameter octagons arranged pair-wise in six blocks, one exposed to elevated CO_2 and one at ambient CO_2 (6 octagons with and 6 without pipes, paired in blocks two and two). Each octagon comprised four plots with the treatments drought or elevated temperature solely or in combination, and a non-warmed, non-drought plot. The temperature was increased by passive nighttime warming, by means of low automatic curtains automatically removed during rain events. The precipitation was altered also

131 with automatic curtains that automatically unfold during rain events. The atmospheric
132 CO_2 was increased with pipe fumigation as in a regular FACE experiment, but with a
133 feed back control system linked to wind speed and wind direction. The temperature
134 increase in 2 cm soil depth was around 1˚C, and the increased CO_2 concentration in the
135 air was 510 ppm. The drought period started in late June 2006 and continued for 5 weeks
136 until early August when soil water reached c. 0.05 m^3m^{-3} water in the top 20 cm of the
137 soil. For further information about the experimental design of the multifactor set up, see
138 Mikkelsen et al. 2008.
139 Each of the 48 plots of the climate treatment experiment had temperature probes
140 installed at 5 cm depth in the soil, at the soil surface, and in the vegetation canopy at 20
141 cm height, each recording temperature on an hourly basis. TDR probes were also
142 installed at 0-20 cm depth and 0-60 cm depth for registration of soil water content on an
143 hourly basis. In addition the water content of the soil samples from the depths 0-5 cm, 5-
144 10 cm and 10-15 was measured once, by drying the soil for two days at 80 ˚C. Cups for
145 collection of precipitation water were installed on two masts at the field site.
146

147 **In situ injection**

148 In each of the 48 plots an area of 80×80 cm^2 was chosen prior to the start of the climate
149 treatments to contain an approximately equal amount of *Calluna vulgaris* (evergreen
150 dwarf shrub) and grasses (mainly *Deschampsia flexuosa* but also *Festuca ovina*). Within
151 each of these areas, a plot of 20×20 cm was labelled with stable isotope $^{15}N^{13}C$-glycine
152 on September 26 2006. The labelling solution was re-demineralised water with ^{15}N and
153 ^{13}C (U-$^{13}C_2$, 98%; ^{15}N 98%) glycine, H_2NCH_2COOH. Each plot received 1 dl of re-

demineralised water with 0.027 g glycine, corresponding to 130 mg N m^{-2}. The label was injected into the soil just below the soil surface with a syringe at 20 evenly distributed points within the 20×20 cm plots.

Plant biomass and soil sampling

One day after labelling, representative shoots from above ground (down to soil surface) vegetation was sampled within the 20×20 cm plots, of *Calluna*, *Deschampsia* (including leaf meristem) and mosses (a mixture of species). Additionally, one day after labelling, soil cores were sampled from the soil surface (including the litter layer) and down to 15 cm depth. Three soil cores were taken from each plot and divided into three soil depths: 0-5 cm, 5-10 cm and 10-15 cm. The subsamples were mixed to a composite sample from each depth and immediately sorted into soil and roots. The samples were kept cold on ice. All plant material (roots and shoots) was washed with 0.5 mM $CaCl_2$, frozen and freeze dried. Within 48 hours, a subsample of the fresh soil from each plot was extracted with re-demineralised water (1:5) on a shaker for 1 hr. and another set of subsamples was vacuum-incubated with chloroform for 24 hrs to release microbial C and N (Joergensen & Mueller, 1996; Brookes *et al.*, 1985) before extraction with water as above. A third subsample of the sorted and sifted soil was freeze dried and used for estimating soil water content. Just before the labelling was performed, additional soil samples and plant shoot samples were taken in adjacent subplots within the climate treated plots to obtain ^{15}N and ^{13}C natural abundances from all the investigated fractions. The same procedures as for the labelled samples were followed with caution not to inter-contaminate with ^{13}C and ^{15}N labelled samples.

One week after labelling, all the remaining aboveground plant material was sampled from the plots in order to obtain plant biomass estimates. The *Calluna* material was sorted into green shoots with green leaves attached, coarse (non-green) branches, coarse roots (> 0.5 mm) and fine roots (< 0.5 mm) and the grasses were sorted into leaves, coarse (> 1 mm), and fine roots (< 1 mm). Mosses and aboveground litter (mainly of grasses, but also of *Calluna*) constituted additional fractions.

Chemical and isotopic analysis

The soil extracts were spectrophotometrically analyzed for NH_4^+ (indophenol-blue reaction) with a Hitachi U 2010 spectrophotometer and for NO_3^- with a Tecator FIAstar analyzer. Part of the extract was digested with H_2SeO_3, H_2SO_4 and H_2O_2 and analyzed as above to yield total dissolved N (TDN), with DON (dissolved organic nitrogen) = TDN – total mineral N. Total microbial N (MicN) was calculated as TDN in the fumigated samples minus TDN in the non-fumigated samples, using 0.4 as the extractability factor (Jonasson *et al.*, 1996; Michelsen *et al.*, 1999; Schmidt *et al.*, 1999). Another part of the extract was analyzed for organic carbon (DOC) with a Shimadzu TOC 5000A analyzer. Total microbial C (MicC) was calculated as DOC in the fumigated samples minus DOC in the non-fumigated samples, using 0.45 as the extractability factor (Schmidt et al., 2000).

For the $^{15}N/^{14}N$ and $^{13}C/^{12}C$ isotope ratio analysis of the fumigated and non fumigated soil extracts, the extracts were freeze-dried in a small bottle containing a quartz filter (Quartz microfibre filters QMA Whatman) and with a parafilm lid with a small hole. Filters, dried crushed soil and plant material were analyzed with a Eurovector

198 CN analyzer coupled to an Isoprime isotope ratio mass spectrometer. Plant material
199 calibrated against certified IAEA standards was used as working standards.

200 **Calculations and statistics**

201 The ^{15}N enrichment of the plant material is reported as excess mole per gN of the
202 material and ^{15}N and ^{13}C enrichments of the microbial biomass is reported as mole per m^{-2} in, excess of natural abundance ^{15}N and ^{13}C (Fry, 2006).. In particular the CO_2 enriched
204 plots exhibited a change in ^{13}C natural abundance, thus for all treatment combinations
205 and each plant or soil fraction, the measured ^{15}N or ^{13}C contents were subtracted with
206 values for each sample component. The ^{15}N recovery was calculated as the percentage of
207 total added ^{15}N label per m^2 recovered in the total dissolved N (TDN), total microbial N
208 (MicN), total soil N pool and in the plant biomass pr m^2.

209 Linear mixed models were applied to analyse the responses using SAS 8.0.
210 Random effect terms were block, treatment plot and octagons, respecting the nested
211 structure of the design. Main effects terms were the treatment factors: CO_2, Temperature
212 (T), and Drought (D). All interaction terms between the factors CO_2, D and T were
213 included. The models were gradually simplified, starting with the third order interaction,
214 taking out non-significant terms until only significant ($P<0.05$) or close to significant
215 ($0.05<P<0.10$) terms remained. Homogeneity of variances was investigated with residual
216 plots and appropriate transformations done if necessary (SAS Institute Inc., 2003).

217 ## *Results*

218 A minor part, 2.4 – 4.7 % of added ^{15}N was recovered in plants one day after labelling,
219 while 43 – 120 % was recovered in microbes (Table 1).

220 The ^{13}C enrichment (Fig. 1) and recovery of ^{15}N in the microbial biomass (Table
221 1) overall decreased significantly (both $P<0.0001$) with depth, with the largest ^{13}C
222 enrichment in the top 0-5 cm depth layer, 30-fold higher than at 10-15 cm depth (Figure
223 1). In 0-5cm depth the overall microbial acquisition of ^{15}N and ^{13}C from glycine
224 correlated significantly, with $^{13}C = 1.74*^{15}N$, $R^2=0.92175$ and $P<0.0001$) (Figure 2).
225 There was a tendency to an interaction effect of the three climate factors at 5-10 cm depth
226 for microbial ^{13}C enrichment ($T*D*CO_2$ P=0.0639).
227 The ^{13}C enrichment in the dissolved organic carbon ($DO^{13}C$) (Figure 3) and ^{15}N
228 recovery of DON (Table 1) also decreased significantly ($DO^{13}C$: $P<0.0001$, $DO^{15}N$:
229 P=0.0393) with depth (Figure 3). In the top 0-5 cm depth layer, CO_2 increased the ^{13}C
230 enrichment of DOC (P=0.0463), mainly in the plots with all treatments combined,
231 causing the significant interaction ($T*D*CO_2$: P=0.0366). Also, drought seemed to
232 decrease ^{13}DOC, but not when combined with warming, causing the highly significant
233 T*D interaction. At 5-10 cm depth, CO_2 tended to decrease ^{13}C enrichment in DOC while
234 warming increased ^{13}C in DOC (CO_2: P=0.0627, $T*CO_2$: P=0.0809, T: P=0.0250).
235 Plant acquisition of the glycine label was seen as both shoot and root ^{15}N
236 enrichment (Table 1 and Figure 4). Only mosses showed an effect of treatment with D:
237 P=0.0006 and $D*CO_2$: P=0.0004, with more ^{15}N enrichment in non-drought plots than in
238 drought plots perhaps because the mosses were in a stage of post-drought hibernation.
239 Some shoot and root samples also showed ^{13}C enrichment, but overall this was non-
240 significant and the results are not presented.
241 For *Deschampsia* fine roots in 0-5 cm depth the model interactions $T*CO_2$ and
242 $T*D*CO_2$ (P=0.0886 and P=0.0486 respectively) was due to a large plant root ^{15}N

243 acquisition in T and in CO_2 plots, which was a non-additive effect (Figure 4a). In 5-10
244 cm depth the model interaction T*D*CO_2 by tendency (P=0.0527) covered a markedly
245 larger ^{15}N acquisition in the +CO_2 plots alone and in the plots with all three treatments
246 combined (Figure 4a). The *Deschampsia* fine root ^{15}N enrichment showed no overall
247 effect of depth.

248 The *Calluna* fine root ^{15}N enrichment overall decreased (P=0.0002) with depth
249 (Figure 4b). In 0-5 cm depth the decreased ^{15}N enrichment with both D and T alone was
250 counteracted when the two treatments were combined, also in combination with CO_2
251 (T*D: P=0.0578). In 5-10 cm depth the model interactions T*D*CO_2 (P=0.0202) and
252 T*D (P=0.0910) covered a decrease in ^{15}N enrichment with warming, except when all
253 treatments were combined (Figure 4b).

254 The *Deschampsia* and *Calluna* root biomasses decreased significantly (both:
255 P<0.0001) by depth (Table 2). The *Deschampsia* fine root biomass at 0-15 cm depth was
256 ten-fold larger than *Calluna* fine root biomass (Figure 5) but the total biomasses of the
257 two species were approximately equal (Table 2). Despite this, the aboveground leaf
258 biomass of *Calluna* at this time of the year generally exceeded that of *Deschampsia*
259 (Figure 6). Across treatments, there was an overall negative effect of warming on fine
260 root biomass of *Deschampsia* (P=0.0305), but no effect on *Calluna* fine root biomass
261 (Figure 5).

262 Warming had a negative effect on aboveground grass (mainly *Deschampsia*) leaf
263 biomass in non-CO_2 plots, while warming promoted grass leaf growth in +CO_2 plots, as
264 shown by the significant T*CO_2 effect (P=0.0247) (Figure 6). For *Calluna* leaf biomass
265 the T*CO_2 interaction tended to have the opposite direction (T*CO_2: P=0.0578). The

266 ratio of leaf to branch in *Calluna*, which presumably is the most response-sensitive
267 biomass variable as it normalizes recent plant production relative to pre-treatment
268 biomass in harvested plot, also showed this significant T*CO_2 interaction (P=0.0038),
269 with higher production relative to old biomass in warmed and in +CO_2 plots, but lower
270 response than expected in the combined T and CO_2 treatments (Figure 6).

271 *Deschampsia* and *Calluna* fine root N concentration decreased significantly with
272 depth (both P< 0.0001). *Deschampsia* fine root N concentration at 10 -15 cm depth
273 increased by warming, (P=0.0139), by contrast, *Calluna* fine roots decreased (P=0.0392),
274 and coarse roots tended to decrease (P=0.0769) by warming in 0-5 cm depth (Table 2).
275 The moss and grass shoot N concentration was not significantly affected by treatment
276 (Table 2), but *Calluna* shoots showed significant effects of treatments in the green
277 fraction, with less N concentration in all CO_2 plots except the one with all treatments
278 combined, as shown by the model interactions T*CO_2: P=0.0276, D*CO_2: P=0.0657 and
279 T*D*CO_2: P=0.0281 (Table 2).

280 Dissolved organic C (DOC) and NH_4^+-N (but not NO_3^--N) decreased, and
281 dissolved organic N (DON) increased with depth (DOC: P=0.0040, NH_4^+-N: P<0.0001,
282 DON: P<0.0001) (Table 2). DOC had a significant effect of treatment in 0-5 cm depth
283 with D*CO_2: P=0.0143 and in 5-10 cm depth with: T*CO_2: 0.0819 (Table 2). At 5-10 cm
284 depth NO_3^--N concentration was lower in response to CO_2 (CO_2: P=0.0106) and higher in
285 response to warming (T: P=0.0691) (Table 2).

286 Microbial biomass C and microbial N decreased with depth (both P<0.0001)
287 (Table 2). Microbial C:N ratio increased with depth (P=0.0038), with no effects of
288 treatment (data not shown). Microbial C showed tendencies towards effects of treatment,

289 with D*CO_2: P=0.0550 in 0-5 cm depth, and T*CO_2: P=0.0620 and T*D: P=0.0824 in
290 10-15 cm depth.
291 The local climate in the week of the labelling experiment (Sept. 23rd to 27th 2006)
292 was stable (Figure 7). The temperature drop from 26th to 27th and the slight increase in
293 soil water content was caused by the 5.2 mm rainfall right after the labelling. At the day
294 of labelling, warming increased the canopy temperature and the soil temperature at 0 cm
295 and 20 cm depth by 0.8, 0.8 and 0.7 ˚C, respectively (all P<0.001). The soil water content
296 showed a tendency to an effect of the preceding drought in 0-20 cm depth and 0-60 cm
297 depth with slight decreases of 0.011 and 0.008 m^3 m^{-3} respectively (P<0.1). The water
298 content in the soil samples taken from 0-5, 5-10 and 10-15 cm depth (Figure 7) was not
299 significantly affected by the climate treatments.
300

301 ## *Discussion*

302 The soil humidity was stable over the period, and at the day of the labelling it was even
303 over the different treated plots. Hence, it is reasonable to assume that the distribution and
304 adsorption of the glycine label was even over all plots. The glycine concentration
305 abundant in the soil prior to labelling was presumably close to that previously measured
306 one year earlier at the field site: 0.197 µgN g-1 SOM ± 0.052 (Andresen et al.,
307 submitted). Hence, as in other heathlands (Abuarghub & Read, 1988; Kielland *et al.*,
308 2006; Sorensen *et al.*, 2007) glycine was present in the soil solution with a low
309 concentration, and our intention of investigating natural glycine acquisition potential by
310 plants and soil microorganisms was justified.

311 The large acquisition of glycine label by the soil microorganisms (36-110 % ^{15}N
312 recovery in 0-5 cm depth) compared to the low acquisition by the plants (2.4 to 4.7 % ^{15}N
313 recovery) was expected from other investigations (Andresen et al, submitted (Hobbie &
314 Chapin III, 1998; Andresen & Michelsen, 2005; Hofmockel *et al.*, 2007; Sorensen *et al.*,
315 2008b; Sorensen *et al.*, 2008a). Hence, in this short term investigation the soil
316 microorganisms rapidly acquired the large part of the added glycine. There was no
317 significant ^{15}N:^{13}C relationship in grass and *Calluna* roots, suggesting that glycine was
318 not acquired as an intact compound by plants, or that ^{13}C was so quickly respired that
319 intact uptake could not be proven *although uptake in intact form has been shown*
320 *previously* (Persson & Näsholm, 2001; Andresen & Michelsen, 2005; Rains & Bledsoe,
321 2007).
322 The decreasing ^{15}N enrichment of plant roots with greater depth was accompanied
323 by the decreasing ^{13}C and ^{15}N enrichment of the microbial biomass and of dissolved
324 organic C and N, indicating a decreasing concentration of the added label downwards,
325 below the surface injection points. Furthermore, the decreasing plant root biomass and
326 soil microbial biomass, and the increasing microbial C:N ratio downwards, together with
327 increasing dissolved organic compounds and NH_4^+-N concentration with greater depth,
328 suggest a downwards decrease in live biomass and altered function with decreased
329 utilization of the organic substrates.
330 The microbial acquisition of ^{15}N and ^{13}C from glycine with the average ratio of
331 1.74, suggest that glycine was acquired by soil microorganisms as intact compounds. A
332 similar microbial ^{15}N ^{13}C glycine acquisition ratio (1.62) has been found in a springtime
333 investigation at the same field site (Andresen et al., submitted). Hence, we conclude that

334 soil microorganisms at this heath acquire glycine as intact compounds, similar to
335 findings in other ecosystem types (Nordin *et al.*, 2004; Näsholm & Persson, 2001;
336 Harrison *et al.*, 2008).

337 The stable microbial acquisition of the glycine label across treatment, suggest that
338 microbial glycine acquisition was not affected by the climate change factors. This lack of
339 response to warming was also found in microbial uptake of $^{15}N^{13}C$ glycine at a subarctic
340 heath (Sorensen *et al.*, 2008b). However, the tendency to microbial biomass C response
341 in this study and significant responses to warming in microbial biomass C and N in a
342 study of soils below *Calluna* separately (manuscript 3),suggest that the soil
343 microorganisms did respond to the treatments, confirming previous observations in
344 heathland soils exposed to drought and warming (Jensen *et al.*, 2003; Sowerby *et al.*,
345 2005), although not with changed potential for acquisition of glycine.

346 The treatment and species specific plant biomass and N concentration responses
347 may be seen as different stages of seasonal development, altered by the climate
348 treatments. In this fashion the green *Deschampsia* leaf and root biomass decrease in
349 response to warming and the deep root N concentration increase in response to warming
350 may be an early seasonal development of *Deschampsia* in response to warming. The
351 contrasting increase in *Calluna* biomass could also reflect an aboveground competition
352 component, at this stage with warming in favour of *Calluna*, and the belowground
353 decrease in *Calluna* root N%, could reflect a belowground competition component, at this
354 stage with warming in favour of *Deschampsia*. In subarctic heath ecosystems parallel
355 increases in shrub biomass but not in herb biomass has been found in response to
356 warming (Sorensen *et al.*, 2008b) while no such changes were observed in Alaskan

357 tundra or remained stable (Hobbie & Chapin III, 1998). *Calluna* growth and leaf N
358 concentration increased in response to warming at a near by heath (Peñuelas *et al.*, 2004)
359 and *Calluna* shoot length and N% increased in response to warming and in response to
360 drought in UK (Gordon *et al.*, 1999), supporting our findings.

361 The increased *Deschampsia* N concentration in response to T may reflect a larger
362 N acquisition, as is also suggested by the larger root ^{15}N acquisition (seen with a soil
363 depth displacement of the acquired label in direction of xylem flow). With the ^{15}N
364 acquisition normalized to $g^{-1}N$ in the root, the larger acquisition truly reflects a positive
365 physiological response to warming and to elevated CO_2. In an experiment with uptake of
366 the amino acid alanine in a pine forest ecosystem under elevated CO_2, a suppressing
367 effect of CO_2 was observed on alanine acquisition (Hofmockel *et al.*, 2007). However,
368 other CO_2 experiments show species specific changes in plant root nitrogen acquisition
369 (Bassirirad, 2000). Increased soil temperature most often increase plant root nutrient
370 uptake, by the mechanism of temperature control of uptake kinetics (Hobbie & Chapin
371 III, 1998; Bassirirad, 2000), in line with the response in our experiment.

372 We interpret the decreased *Calluna* leaf N concentration response to CO_2 as a
373 carbon dilution effect, presumably caused by increased photosynthetic carbon acquisition
374 in CO_2 plots, as suggested by the increase in *Calluna* leaf biomass and leaf/branch ratio
375 in CO_2 plots, and by the *Calluna* root ^{15}N acquisition being non-responsive to CO_2.
376 Likewise, the soil NO_3^--N decrease in response to CO_2 could reflect increased plant
377 nitrogen N acquisition. The large *Deschampsia* ^{15}N root acquisition in CO_2 plots could
378 reflect an increased plant N acquisition in response to increased growth. However,
379 increased green leaf biomass was not seen at the time of sampling, although the dilution

of N in *Deschampsia* leaves seemed to suggest that such an effect was taking place at this time of peak plant biomass. Other studies have found species specific increased root biomass in response to warming and elevated CO_2 (Volder *et al.*, 2007) or no response in root biomass but elevated starch concentration in response to elevated CO_2 (Handa *et al.*, 2008). The lack of biomass CO_2 effect in our experiment may be caused by the short CO_2 fumigation period and possibly seasonal changes at the time of sampling in line with the large variability (Bassirirad, 2000).

Conclusions

The climate change factors significantly caused physiological-ecological changes in the temperate heathland ecosystem. Soil microorganisms acquired the largest part of the added glycine and acquired intact compounds with no significant effects of treatment. *Deschampsia* and *Calluna* plants also acquired glycine, with no proof of intact acquisition. *Deschampsia* fine root biomass decreased in warmed plots reflected by larger nitrate concentration in the sub-soil. Large *Deschampsia* plant root ^{15}N acquisition in T and in CO_2 plots met our hypothesis of promoted plant N demand, when plant biomass increased, but this was a non-additive effect. *Deschampsia* green leaf biomass decreased in warmed plots but not when CO_2 was added, and *Calluna* green to coarse branch increased in warmed plots and in elevated CO_2 plots, but not when these treatments were combined. Hence, the responses to simulated increased root exudation in form of ^{15}N $^{13}C_2$-glycine were significant and non-additive.

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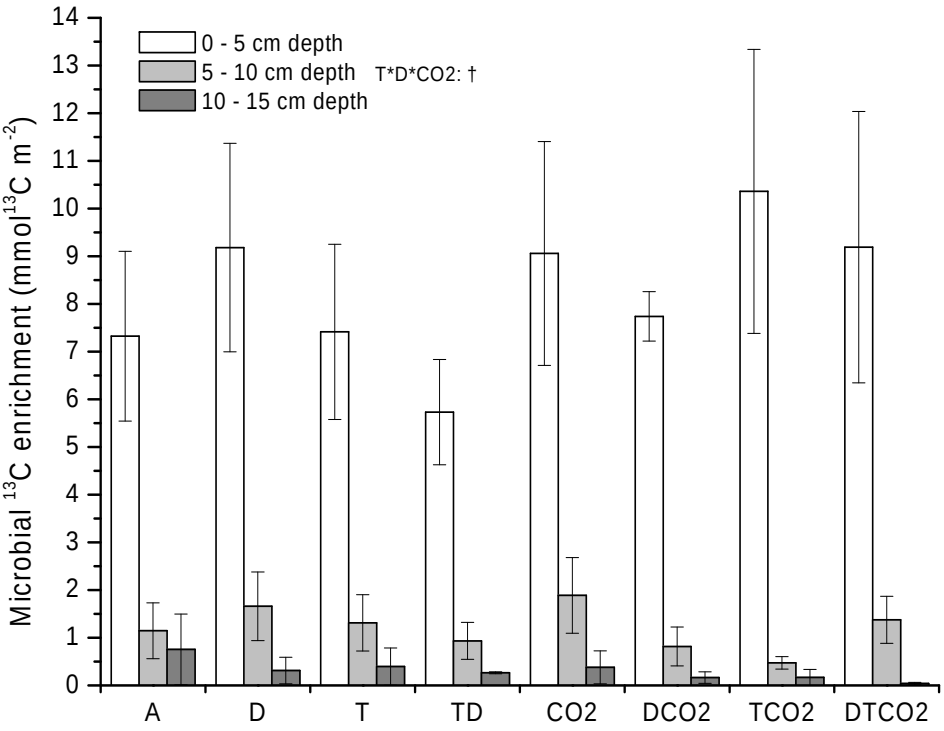

Figure 1: Microbial carbon ^{13}C enrichment (mmol ^{13}C m^{-2}) of $^{15}N^{13}C_2$-glycine labelled chloroform fumigated extracted soil samples from 0-5 cm, 5-10 cm and 10-15 cm depth. Statistical significant effects from proc mixed model analysis of variances for the main effects: D, T and CO2 and the interactions D*T, D*CO2, T*CO2 and D*T*CO2 is indicated as follows: *** indicates $P < 0.001$; ** indicates $P < 0.01$; *: $P < 0.05$; †: $P < 0.1$.

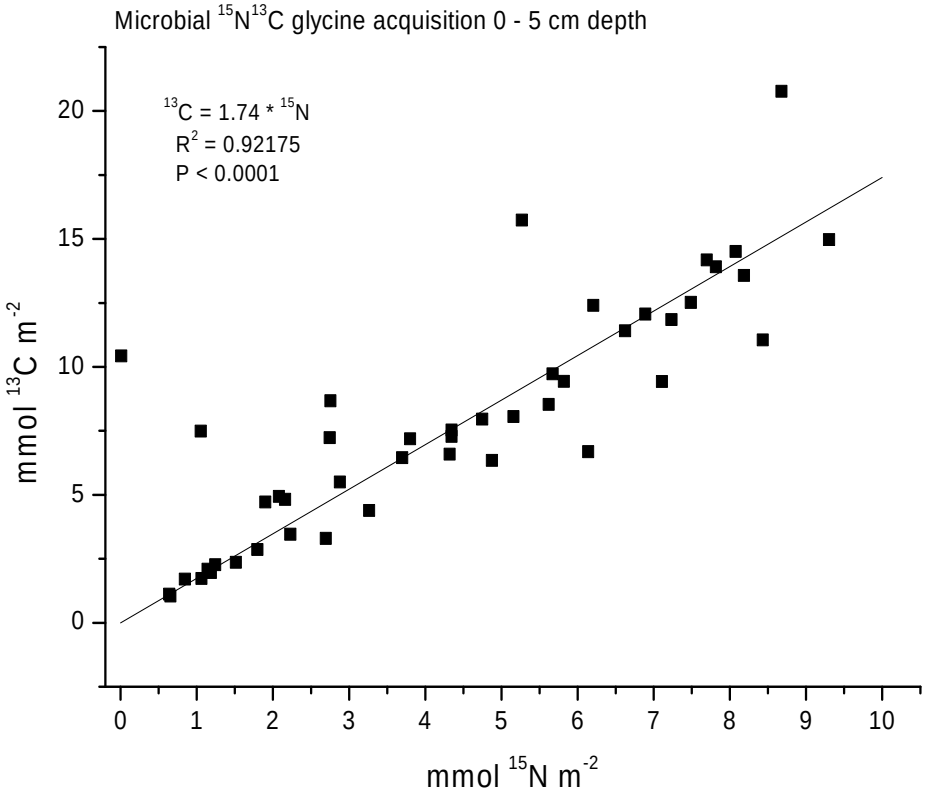

Figure 2: ^{15}N enrichment (mmol^{15}N m^{-2}) versus ^{13}C enrichment (mmol^{13}C m^{-2}) in microbial biomass sampled at 0-5 cm depth one day after labelling with $^{15}N^{13}C_2$-glycine Linear regression forced through zero, all climate treatments (no significant effects), n = 48.

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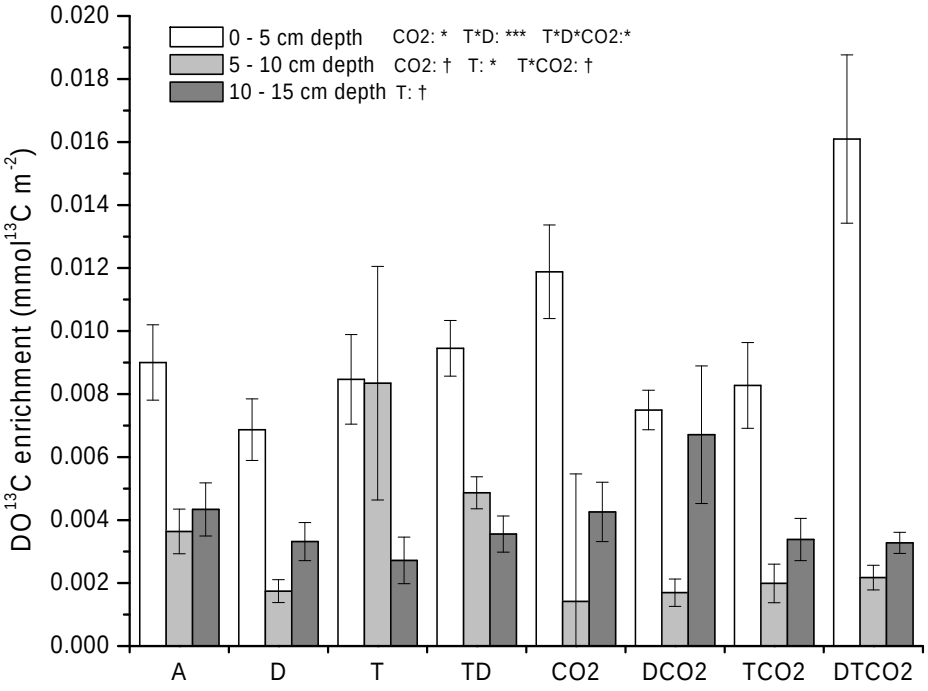
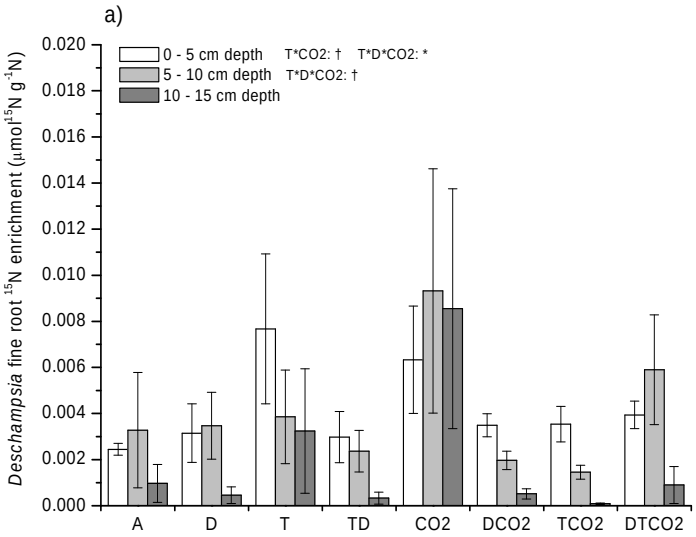


574

575 **Figure 3:** Dissolved organic carbon ^{13}C enrichment (mmol ^{13}C m^{-2}) of $^{15}N^{13}C_2$-glycine
576 labelled extracted soil samples from 0-5 cm, 5-10 cm and 10-15 cm depth. Statistical
577 significant effects from proc mixed model analysis of variances for the main effects: D, T
578 and CO2 and the interactions D*T, D*CO2, T*CO2 and D*T*CO2 is indicated as
579 follows: *** indicates $P < 0.001$; ** indicates $P < 0.01$; *: $P < 0.05$; †: $P < 0.1$.

580



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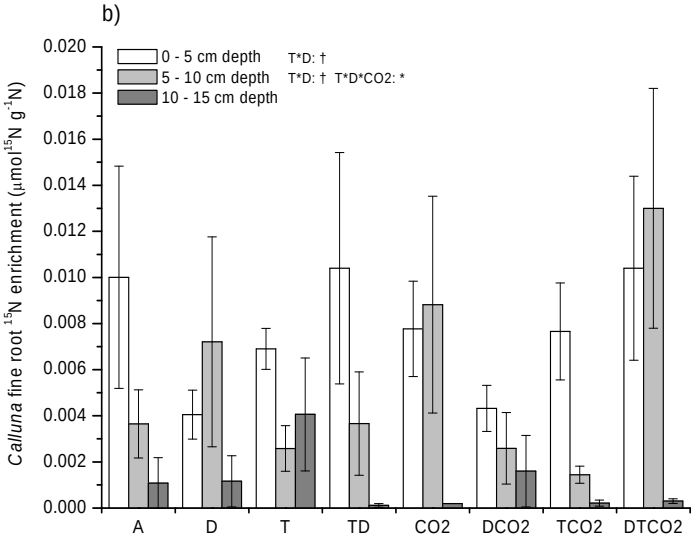


582

583 **Figure 4: a)** *Deschampsia* and *Calluna* **b)** fine root ^{15}N enrichment (µmol ^{15}N $g^{-1}N$).
584 Statistical significant effects from proc mixed model analysis of variances for the main
585 effects: D, T and CO2 and the interactions D*T, D*CO2, T*CO2 and D*T*CO2 is
586 indicated as follows: *** indicates P < 0.001; ** indicates P < 0.01; *: P < 0.05; †: P <
587 0.1.

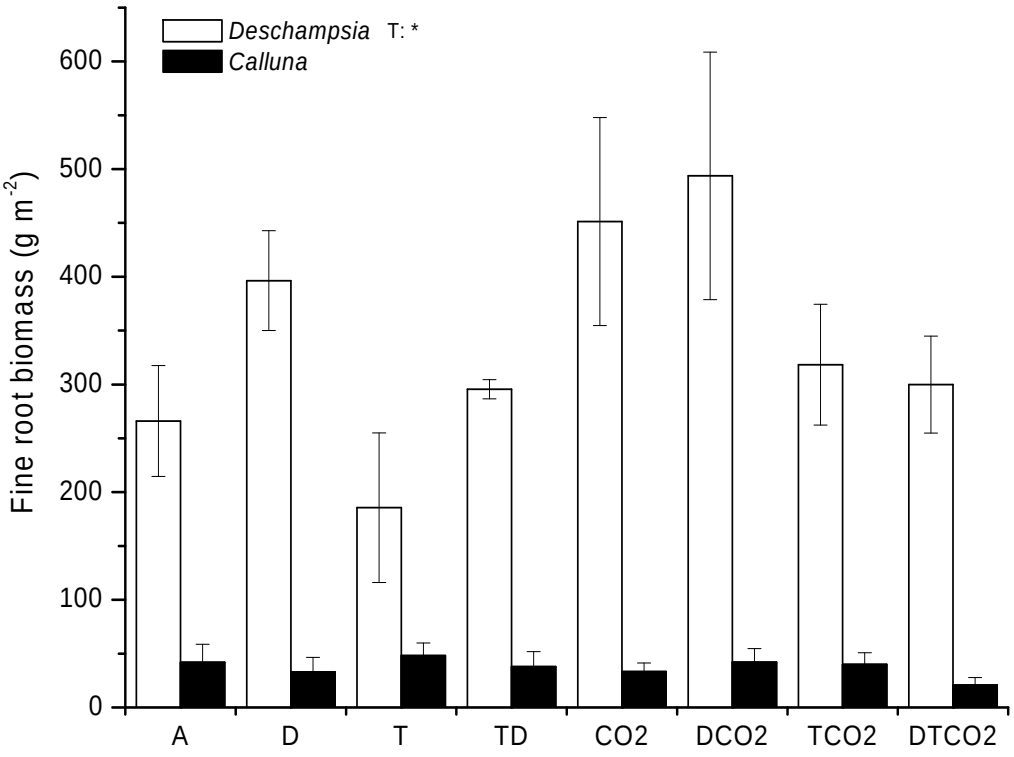
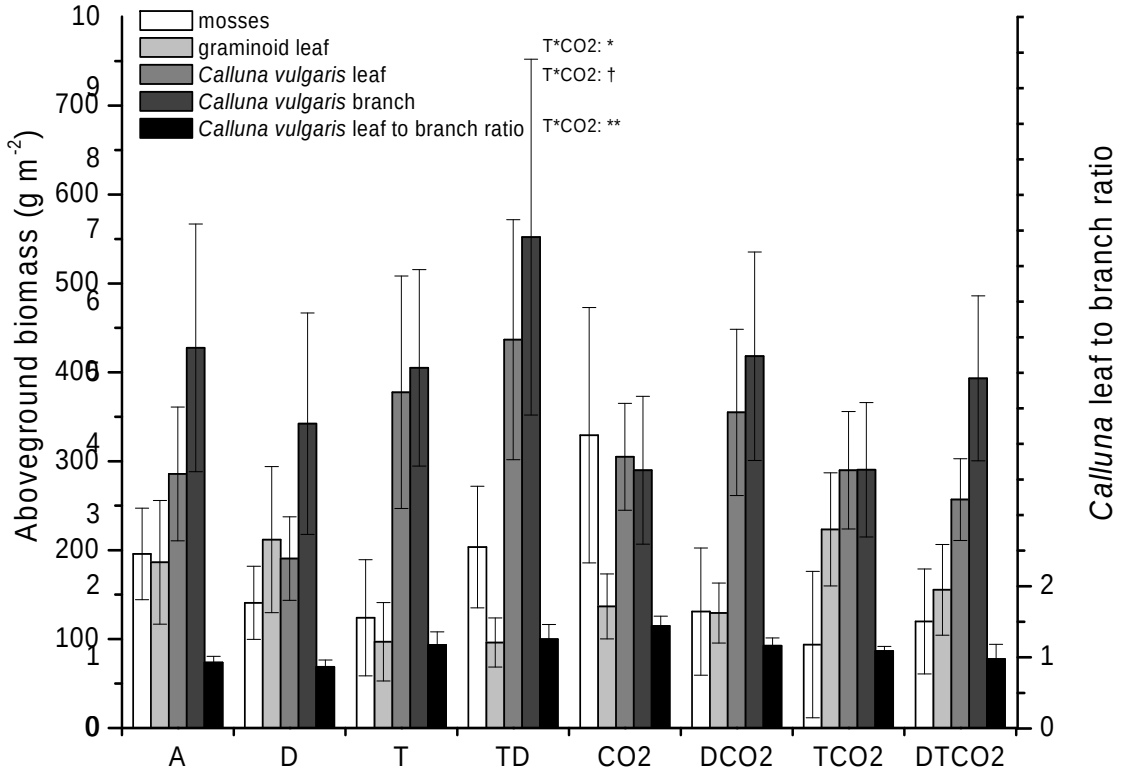


Figure 5: Fine root biomass in g m^{-2} of grasses (open bars) and *Calluna* (dark bars) summed from 0 to 15 cm depth (mean and standard error). Statistical significant effect from proc mixed model analysis of variances for the main effect of T; *: $P < 0.05$.



601

602 **Figure 6:** Aboveground plant biomass at 27th September 2006 harvested in 20×20 cm
603 plots. Plant fractions: mosses, graminoid green leaf, *Calluna* green leaf, *Calluna* branch,
604 and *Calluna* leaf to branch ratio (right hand scale). Statistical significant effects from
605 proc mixed model analysis of variances for the main effects: D, T and CO2 and the
606 interactions D*T, D*CO2, T*CO2 and D*T*CO2 is indicated as follows: *** indicates
607 P < 0.001; ** indicates P < 0.01; *: P < 0.05; †: P < 0.1.

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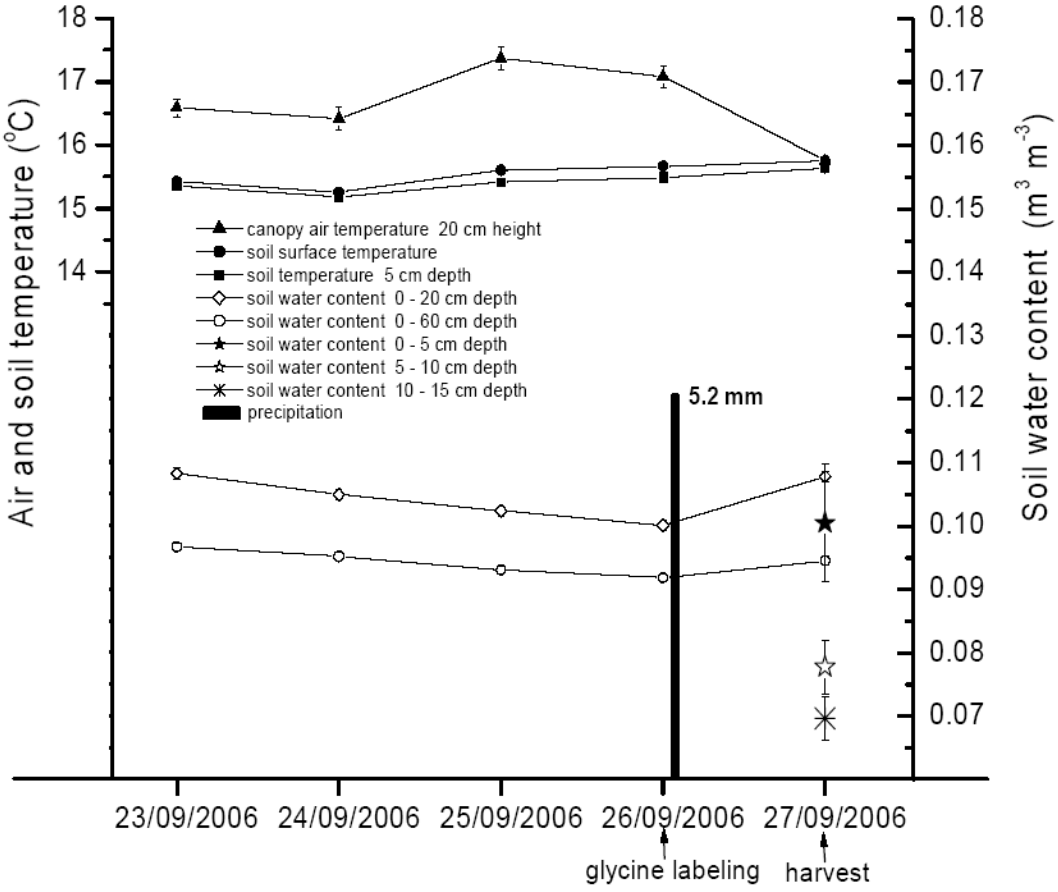

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611

612 **Figure 7:** Field site temperature of air and soil and soil water content in the week up to
613 the glycine labelling, mean and standard error over all 48 treatment plots. Air, soil surface
614 and soil (5cm depth) temperature (˚C) measured with temperature probes. Soil water
615 content (%) in 0-20 and 0-60 cm depth, measured with TDR probes. 0-5 cm, 5-10 cm and
616 10-15 cm depth water content, measured in soil samples dried at 80 ˚C. Precipitation
617 (mm) during the night 26th to 27th September, mean over two meteorological masts.

618

Table 1: Ecosystem properties after one year of climate treatments. Statistical significant effects from proc mixed model analysis of variances for the main effects: D, T and CO2 and the interactions D*T, D*CO2, T*CO2 and D*T*CO2 are indicated with bold if $P < 0.05$; and with bold italics if $P < 0.1$.

Table 2: ^{15}N recovery (%) in soil microbial biomass N, dissolved organic N and the whole plant (all shoot and root fractions and depths) one day after $^{15}N^{13}C$ glycine labelling. No statistical effects of treatments were found.